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EDITORIAL PREFACE

Jordan Journal of Biological Sciences (JJBS) is a refereed, quarterly international journal financed by the Scientific Research and Innovation Support Fund, Ministry of Higher Education and Scientific Research in cooperation with the Hashemite University, Jordan. JJBS celebrated its 11th commencement this past January, 2019. JJBS was founded in 2008 to create a peer-reviewed journal that publishes high-quality research articles, reviews and short communications on novel and innovative aspects of a wide variety of biological sciences such as cell biology, developmental biology, structural biology, microbiology, entomology, molecular biology, biochemistry, medical biotechnology, biodiversity, ecology, marine biology, plant and animal biology, plant and animal physiology, genomics and bioinformatics.

We have watched the growth and success of JJBS over the years. JJBS has published 11 volumes, 45 issues and 479 articles. JJBS has been indexed by SCOPUS, CABI's Full-Text Repository, EBSCO, Clarivate Analytics- Zoological Record and recently has been included in the UGC India approved journals. JJBS Cite Score has improved from 0.18 in 2015 to 0.60 in 2018.

A group of highly valuable scholars have agreed to serve on the editorial board and this places JJBS in a position of most authoritative on biological sciences. I am honored to have six eminent associate editors from various countries. I am also delighted with our group of international advisory board members coming from 15 countries worldwide for their continuous support of JJBS. With our editorial board's cumulative experience in various fields of biological sciences, this journal brings a substantial representation of biological sciences in different disciplines. Without the service and dedication of our editorial; associate editorial and international advisory board members, JJBS would have never existed.

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Finally, JJBS would not have succeeded without the collaboration of authors and referees. Their work is greatly appreciated. Furthermore, my thanks are also extended to The Hashemite University and the Scientific Research and Innovation Support Fund, Ministry of Higher Education and Scientific Research for their continuous financial and administrative support to JJBS.

Professor Khaled H. Abu-Elteen
March, 2019

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Assessment of *Satureja montana* L. and *Mentha piperita* L. Antioxidant Activity, Cytotoxicity and Pattern of Apoptotic Gene Expression in Hepatoma Cells

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Abstract

This study is intended to assess the *Satureja montana* L. and *Mentha piperita* L. *var. citrata* antiradical potential and elucidate their molecular mechanisms underlying the cytotoxic induction in HepG2 cells. The total phenolic and flavonoids of the selected plants were determined. Their antioxidant activity was evaluated using 2, 2-diphenyl-1-picryl-hydrazylhydrate ($DPPH^{\cdot}$), 2,2'-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid ($ABTS^{+)}$ radicals and reducing power. The HepG2 cells were incubated with tested compounds at different concentrations ranging between 12.5 and 200µg/mL. Cytotoxicity was measured by MTT and the mRNA expression of Bcl-2, Bax and caspase-3 genes were estimated using Semi-quantitative RT-PCR. The results revealed that the *S. montana* and *M. piperita* aqueous extracts have high content of phenolic 188.2 ± 1.3 and 183.2 ± 0.9 mg GA/g and flavonoid 95.01±2.29 and 144.13±0.73 mg quercetin/g compounds. While their essential oils contain phenolic compounds 290.8 ± 1.4 and 9.2 ± 5.6 mg GA/g only. *In vitro* assays revealed a strong radical scavenging potential of the *S. montana* L. and *M. piperita* L. essential oils against stable $DPPH^{\cdot}$, $ABTS^{+}$ and reducing power showing the highest radical quenching activity compared with the aqueous extracts. In addition, the extracts and oils of the two tested plants showed a concentration-dependent increase in growth inhibition against HepG2 cell line. Semi-quantitative RT-PCR established that either *S. montana* or *M. piperita* up-regulated HepG2 Bax and caspase-3 mRNA expression and down-regulated Bcl-2. Greater variation in Bax, Bcl-2 and caspase-3 expression were visible in the oils rather than the extracts. The decrease in Bcl-2, rise in Bax and lastly, the stimulation of caspase-3 in HepG2 cells indicated the irreversible apoptotic process. Accordingly, the results confirmed that selected plants have an antioxidant potential and apoptosis ability via the activation of mitochondrial apoptotic pathways.

Keywords: *Satureja montana* L, *Mentha piperita* L, Hepatocellular carcinoma, Antioxidants activity, Total phenols and flavinoids, Cytotoxicity, Gene expression.

1. Introduction

The dominant form of primary liver tumor is Hepato-cellular carcinoma (HCC) and is a leading cause of cancer-related death (Balogh *et al*., 2016). Approximately 90 % of patients with HCC have a confirmed background of a chronic liver disease including hepatitis B and C virus chronic infections (El-Serag and Rudolph, 2007; Poon *et al*., 2009). Several treatment options are obtainable against HCC including therapeutic resection, radiofrequency ablation, liver transplantation, trans-arterial chemo-embolization and radio-embolization (Raza and Sood, 2014). The main scientific challenge confronting HCC is the limited response to chemotherapy and the resistance during the treatment due to an improved DNA repair ability and antioxidant enzymes activated in the malignant cells (Thomas, 2009). Recently, several studies have been made with folk medicine for the treatment of cancer. Traditional medicine has been utilized with chemotherapy and radiotherapy to restrain the noxious effects of them and improving overall efficiency (Ling et *al*., 2014).

The *Lamiaceae* family plants are vital ornamental aromatic plants, many of which possess essential oils that are used in medicine, food, cosmetics, and the pharmaceutical industry (Benabdallah *et al*., 2016). The medical and pharmaceutical importance of essential oils are ascribed to their protective activity against the reactive oxygen species (ROS) oxidative damage (Spiridon *et al*., 2011). The *Lamiaceae* family medicinal plants antioxidant capacity can be attributed to their polyphenolic compound content (Adaszynska and Dzieciol, 2017).

Satureja montana L. is generally known as winter savoury and is habitually used for diuretic, bronchitis, curing greasy skin and sensitivity antitussive, antidiarrheal agent stomachic, antiseptic muscle pain, wounds, urinary system infections, and gastroenteritis (Hadian *et al*., 2012; Lerebour *et al*., 2014; Jafari *et al*., 2016). *Satureja* species secondary metabolites, essential oils, phenolic compounds,

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and flavonoids are recognized for their healing properties. Plant extracts or essential oils from *Satureja* various species have revealed several biological activities (Vagionas *et al*., 2007; Grosso *et al*., 2009; Dunkic *et al*., 2010). Mediterranean basin wild species have shown a cytotoxic property against cancer cells (Chinou *et al*., 2007; Menichini *et al*., 2009; Nemati *et al*., 2018). In addition, the *S. montana* essential oil represents an appropriate agent for applications in the fortification of free radical-mediated oxidative stress biological matrices (Trifan *et al*., 2015).

Mentha piperita L. is usually known as peppermint. It is a natural hybrid of *M. aquatica* L. and *M. spicata* L. The entire herb of *M. piperita* is used in phytotherapy for their proven antibacterial, antioxidant, cytotoxic and antiallergenic activities (Capuzzo and Maffei, 2016). Furthermore, the *Mentha* species are known as good free radical scavengers that limit ROS attack on biological and food systems (Sytar *et al*., 2018). In addition, it has been found that various plants which belong to the family of *Lamiaceae*, such as *Origanum vulgare*, *O. majorana*, *Salvia fruticosa*, *S. officinalis* and *Melissa officinalis* provoke apoptosis in the tested malignant cells which plays an essential task in the elimination of injured or malignant cells (Abdel-Massih *et al*., 2010; Darzi and Amirghofran, 2016).

Therefore, the target of the current study is to inspect the *S. montana* L. and *M. piperita* L. extracts' and essential oils' phenolic and flavonoids total content, antioxidant activity and their growth inhibition ability on the HepG2 cell line. The study also presents an assessment of their effect on apoptotic gene expression pattern.

2. Materials and Methods

2.1. Chemicals

Potassium ferricyanide K_3 [Fe $(CN)_6$], ferric chloride ($FeCl_3$), trichloroacetic acid (TCA), peroxidase, hydrogen peroxide, 2, 2'-azino-bis (3-ethylbenz-thiazoline-6-sulfonic acid, diammonium salt (ABTS), 1,1-diphenyl-2-picryl-hydrazyl (DPPH); l- ascorbic acid (vitamin C), Folin & Ciocalteu's phenol reagent were purchased from Sigma-Aldrich (Schnelldorf, Germany). The acetonitrile and water were of the HPLC grade. Dimethylsulphoxide (DMSO), 5-Flurouracil and MTT (3-(4, 5- dimethyl-thiazol-2-yl)-2, 5-diphenyltetrazolium bromide) were purchased from Merck (Darmstadt, Germany). All other chemicals and reagents used in this study were of the analytical grade, and were purchased from Sigma Aldrich chemical Co. (St. Louis, MO, USA). The human liver cancer cell line HepG2 was purchased from VACSERA.

2.2. Plant Materials and Extraction

The study was carried out on *Satureja montana* L. and *Mentha piperita* L. *var. citrata* (Eau de Cologne mint) at the National Research Centre experimental field during two successive seasons (2016 and 2017). The fresh aerial parts were collected at the end of July. The aerial parts were air-dried in a room far from the sun for three weeks. A voucher specimen of the plant is deposited at the National Research Centre herbarium at Dokki, Giza, Egypt.

2.2.1. Essential Oil Isolation

The *S. Montana* L. and *M. piperita* L. *var. citrata* aerial parts were powdered (250 g) and hydro-distilled using a Clevenger type apparatus for three hours according to the method recommended in British Pharmacopoeia. The resulting essential oil was dried over anhydrous sodium sulfate, and was stored at 4°C until its use.

2.2.2. Preparation of Plant Extracts

250 g of the air-dried powdered plant aerial parts of *S. montana* L. and *M. piperita* L. *var. citrata* were extracted by maceration in 1000 mL of distilled water three successive times. The combined extracts were evaporated under reduced pressure at 45°C to the residue, and were stored at 4°C until tested.

2.3. Phenolic and Flavonoid Extracts Content

2.3.1. Total Phenolic Content

The total phenolic content (TPC) of the plants extracts and their essential oils were determined by Folin–Ciocalteu method as described by Singleton *et al*. (1999). The content of phenolics in the extracts was expressed in terms of gallic acid equivalent (mg of GA/g of extract).

2.3.2. Total Flavonoid Content

The flavonoid content of the plants' extracts was determined according to the method of Zhishen *et al.* (1999). The results were expressed as mg quercetin equivalents/g extract.

2.4. *Free Radical Scavenging Activity*

The free radical scavenging activity of the plant extracts and essential oils of *S. montana* L and *M. piperita* L. *var. citrata* was measured by 1, 1-diphenyl-2-picryl-hydrazil (DPPH) using the method of Yamaguchi *et al.* (1998). Briefly, 0.1mM solution of DPPH in ethanol was prepared, then, 1mL of this solution was added to 3 mL of the tested extracts as well as the standard solution at different concentrations (10, 25, 50, 75,100 and 200 µg/ mL). The mixture was shaken vigorously, and was allowed to stand at room temperature for thirty minutes. Then, the absorbance was measured at 517 nm in a spectrophotometer (Jasco, serial No. C317961148, Japan). The lower absorbance of the reaction mixture indicated a higher free radical scavenging activity. The DPPH radical concentration in the reaction medium was calculated via the following equation:

DPPH scavenging effect (%) = $[(A_0\text{-}A_1)/A_0) \times 100]$, Where A_0 is the absorbance of the control reaction, and A_1 is the absorbance in the presence of the sample of extracts.

2.5. ABTS+ Radical Scavenging Activity

$ABTS^+$ radical scavenging activity of the plant extracts, essential oils, and standard were measured according to the method described by Thaipong *et al.* (2006). Each tested sample was prepared in five concentrations (10, 25, 50, 100 and 200µg/mL), and the ascorbic acid was used as standard material with the same concentrations. Exactly 0.2ml of peroxidase (4.4U/ mL), 0.2mL of H_2O_2 (50 µmol/l), 0.2mL of ABTS (100µmol/l), and 1mL methanol were mixed and kept in the dark for one hour to form a bluish green complex after the addition of 1mL of the tested samples and standard at different concentrations. The absorbance at 734nm was measured to represent the

total antioxidant capacity, and was then calculated as follows:

$ABTS^{+}$ radical scavenging activity % = 1- (A sample/ A control)* 100 Where A sample is the absorbance of the samples and standards, and A control is the absorbance of control.

2.6. Reduction Capability

The total ferric-reducing capacity of the plant extracts and essential oils was determined according to the method of Oyaizu (1986). The different concentrations of extracts and oils (10, 25, 50, 75, 100 and 200 µg/ mL) in 1mL of methanol were mixed with phosphate buffer (2.5 mL, 0.2M, pH 6.6) and potassium ferricyanide K_3 [Fe (CN) $_6$] (2.5 mL, 1%). The mixture was incubated at 50˚C for twenty minutes. A portion (2.5 mL) of TCA (10%) was added to the mixture, which was then centrifuged for ten minutes at 1000 rpm (MSE Mistral 2000, UK, and Serial No.: S693/02/444). The upper layer of the solution (2.5mL) was mixed with methanol (2.5mL) and $FeCl_3$ (0.5mL, 0.1%), and the absorbance was measured at 700 nm in a spectrophotometer. l- ascorbic acid was used as a control. The higher absorbance of the reaction mixture indicated a greater reducing power.

2.7. Cell Culture and Treatment

Human liver cancer cell line (HepG2) cells were seeded into standard 96-well culture plates at a density of $2.0x10^4$ cells/well and cultured in RPMI-1640 supplemented with 10% fetal bovine serum (Gibco, Grand Island, NY) and 1% penicillin-streptomycin, at 37°C, in humidified air containing 5 % CO2 to ~70 % confluence. All experiments were performed while the cells were in an exponential growth phase (Lin et *al*., 2011). After the cell adherence, different concentrations of the investigated extract and essential oil ranging between 12.5 and 200µg/mL were added to the wells. The standard reference drug (5-Flurouracil) was added to the cells suspended in 0.10 ml of RPMI medium after washing the cells several times with phosphate buffered saline (PBS). All tests and analyses were run in triplicate, and the incubation continued for forty-eight hours.

2.8. Cytotoxicity Assay

Cytotoxicity was assessed by a tetrazolium-based colorimetric assay (MTT) that measures reduction of tetrazolium salt (3-[4, 5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; Sigma, St. Louis, MO) to formazan, mainly via the activity of mitochondrial enzymes according to Berridge *et al*. (2005). In brief, l0µL of MTT dye solution (5mg/mL) was added to each well, and the plate was incubated for a further four hours at 37°C. After the removal of the MTT dye solution/medium, each well received 100µL DMSO (0.1%), and the absorbance at 490nm was quantified using an ELISA plate reader. The extent of cytotoxicity induced was calculated by comparing absorbance values against those in the control wells. Cytotoxicity was expressed as the concentration of extract or oil inhibiting cell viability by 50% (IC50). The percentage of cytotoxicity was calculated as follows:

% Cytotoxicity = 1- [(OD treated-OD blank) / (OD control-OD blank)] ×100.

2.9. Reverse Transcription-Polymerase Chain Reaction (RT-PCR) and Gene Expression Analysis

Semi q-PCR was performed as described previously by (Rey *et al.,* 2000). The total RNA was isolated from untreated and treated HepG2 cells with TRIzol, and oligo (dT)-primed RNA (1 µg) was reverse-transcribed using SuperScript II reverse transcriptase by RT PreMix Kit (iNtRON Biotechnology, Korea) according to the manufacturer's instructions. The obtained cDNA was used to determine the mRNA expression levels of Bcl-2, Bax and caspase-3 by PCR analysis. GAPDH was used as an internal control. The primer sequences used for the amplification of Bcl-2, Bax, caspase-3 and GAPDH were as follows: Bcl-2-F: 5'-GGATGCCTTTGTGGAACTGT-3', and Bcl-2-R: 5'-AGCCTGCAGCTTTGTTTCAT-3'.Bax–F:5'-TTTGCTTCAGGGTTTCATCC-3',Bax-R:5'-CAGTTGAGTTGCCGTCAGA3'.,caspase-3F:5'-CAGAA GATACCAGTGGAGGCC-3',caspase-3-R:,5'-TTCCG GT TAACACGAGTGAGG-3' and GAPDH- F:5′CAAG GTCATCCATGACAACTTTG-3′, GAPDH-R:5′-GTCC ACCACCCTGTTGCTG TAG -3′. All primers were synthesized by Promega Corp (Madison, WI, USA). The reaction included 1 µL cDNA, 2 µL 10X Taq Buffer, 1.2 µL 25 mM $MgCl_2$, 2 µL 10 mM dNTP, 0.1 µL 5U/µL Taq polymerase, 2 µL each primer and DEPC water up to 20 µL. The PCR conditions were as follows: 95˚C for three minutes and thirty cycles at 95˚C for thirty seconds, 56˚C for forty seconds and 72˚C for forty seconds. The samples were analyzed by 1.5 % agarose gel electrophoresis. The DNA bands were evaluated using a Gel Documentation System (Gel Doc 2000; Bio-Rad Laboratories, Inc., Hercules, CA, USA). The ethidium bromide-stained gel bands were scanned, and the signal intensities were quantified by the computerized Gel-Pro (version 3.1 for window 3). According to the amplification procedure, the relative expression of each gene was calculated using the formula: Optical density of each gene (OD)/ Optical density of GAPDH.

2.10. Statistical Analysis

The values are presented as the mean ± standard error. Results were analyzed by one-way analysis of variance. When significant treatment effects were detected, Dunken-test was performed to make pair wise comparisons between individual means. Statistical analysis software SPSS 16.0 (International Business Machines, Armonk, NY, USA) was used to conduct statistical analyses. $P \leq 0.05$ was considered a statistically significant difference.

3. Results

3.1. Total Phenolic and Flavonoid Content

The *S. montana* L. TPC, calculated from the calibration curve ($R^2 = 0.998$), equal (188.2 ± 1.3) mg gallic acid equivalents/g for the extract and (290.8 ± 1.4) mg gallic acid equivalents/g for the oil, while the TPC of *M. piperita* L. *var. citrata* was 183.2 ± 0.9 & 9.2 ± 5.6 mg gallic acid equivalents/g extract and volatile oil, respectively (Table 1). No significant variation in the TPC of the two plant extracts, while the *S. montana* L. essential oil contains a high significant TPC than the *M. piperita L. var. citrata* In regards to the *S. montana* L., the total

flavonoid content 95.01±2.29 was less than that of *M, piperita L. var. citrata* 144.13±0.73 mg quercetin/g extract. There were no flavonoids in their essential oils (Table 1).

Table 1. Total phenolic and flavonoid compounds in *Satureja montana L.* and *Mentha piperita L.* extracts and essential oils

	Satureja montana L.		*Mentha piperita L.*	
	Extract	Essential oil	Extract	Essential oil
Total Phenolic (mg eq GA/g)	188.2±1.3[a]	290.8±1.4[b]	183. 2±0.9[a]	9.2±5.6[a]
Total Flavonoids (mg eq Que/g)	95.01±2.29b	-----	144.13±0.73a	----

Data are presented as means ± SE. One-way Analysis of Variance was used for data analysis (n=3), Different superscripts within the same raw designate significant differences ($P \leq 0.05$).

3.2. Free Radical-Scavenging Activity

The plant extracts, oils, and ascorbic acid as standard free radical-scavenging activity were measured using the DPPH and ABTS assays. Both DPPH and ABTS that dissolve in methanol or ethanol and are stable free radicals, their colors show special absorptions at 517 nm or 734 nm, respectively. From the DPPH and ABTS^{+} data, the aqueous extracts and essential oils of both plant species exhibited antioxidant activity. When compared with the oxidative potential of the standard compound, remarkable activity was exerted by the essential oils of both plants as presented in Figures 1 and 2.

3.3. Reducing Capacity

The total ferric reducing capacity of an extract may be considered as a significant marker of its possible antioxidant activity. We can see that the reducing power percentage values of S. *montana* L. and *Mentha piperita* L aqueous extracts and volatile oils increased in a concentration-related manner in the range of the tested concentrations (Figure 3). Their relative reducing powers were as follows: volatile oil > aqueous extract.

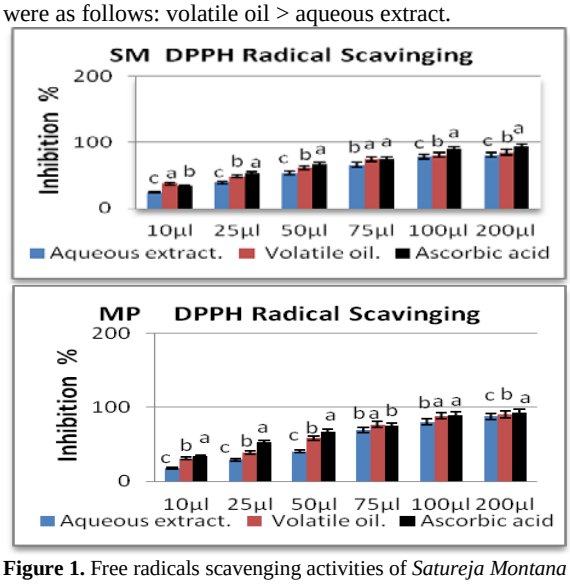

Figure 1. Free radicals scavenging activities of *Satureja Montana* L. and *Mentha piperita* L. *var. citrata* aqueous extracts and essential oils at different concentrations (10-200µg/ml) compared with vitamin C against DPPH. Data are presented as means ± SE. Different superscripts within the same raw designate significant differences ($P \leq 0.05$).

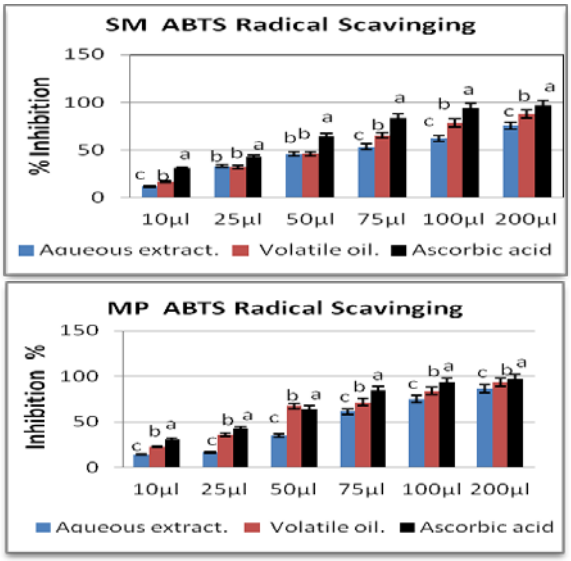

Figure 2. Free radicals scavenging activities of *Satureja Montana* L. and *Mentha piperita* L. *var. citrata* aqueous extracts and essential oils at different concentrations compared with vitamin C against ABTS. Data are presented as means ± SE. Different superscripts within the same raw designate significant differences ($P \leq 0.05$).

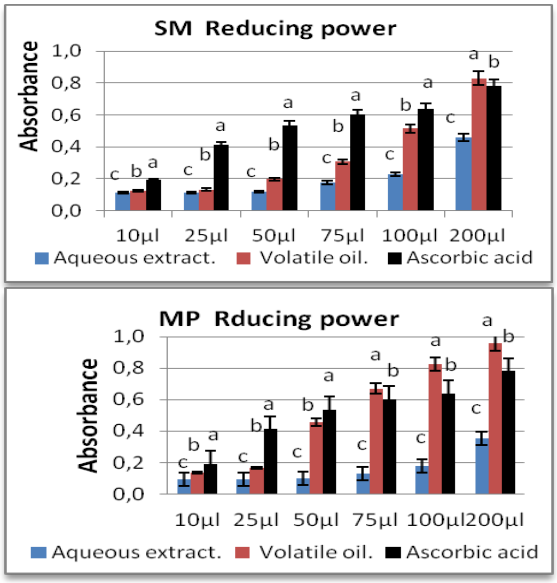

Figure 3. Total ferric reducing power (FRAP) activity of *Satureja montana* L. and *Mentha piperita* L. *var. citrata* aqueous extracts and essential oils at different concentrations compared with vitamin C. Data are presented as means ± SE. Different superscripts within the same raw designate significant differences ($P \leq 0.05$).

3.4. Cytotoxicity

The cytotoxicity of the *S. montana* L. and *M. piperita* L. *var. citrata* aqueous extracts and essential oils was investigated using MTT assay against hepatocellular carcinoma cell lines (HepG2) in comparison with 5-Flurouracil as the reference drug. The results are expressed as the median growth inhibitory concentration (IC_{50}) which is the concentration of the tested compounds that reduces the cells, survival to the half compared with the untreated cells. The results demonstrated that the *S. montana* L. aqueous and oil extracts show cytotoxicity

against the HepG2 cells with IC_{50} value 50 & 25µg/mL, respectively. While, *M. piperita var. citrata* aqueous and oil extracts showed an anticancer effect with IC50 values of 91.5 and 20.2 µg/mL, respectively. However, the *S. montana* L. and *M. piperita* L. *var. citrata* extracts and essential oils cytotoxicity was less than that of the 5-Flurouracil IC50 = 4.5 µg/mL (Figure 4).

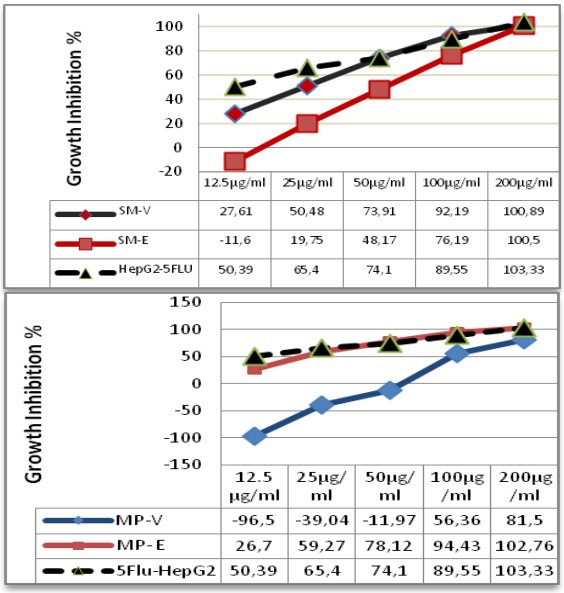

Figure 4. Growth inhibition of *Satureja montana* L. and *Mentha piperita* L. *var. citrata* aqueous extract and volatile oil concentrations against HepG2 cells.

3.5. Effects of Satureja montana L. and Mentha piperita L. Extracts on the Expression of Apoptosis-Related Genes in HepG2 Cells

To find out the molecular mechanisms of the *S. montana* L. and *M. piperita* L. *var. citrata* aqueous extracts and essential oils against the HepG2 cells, the expression of three apoptosis-related genes (caspase-3, Bax and Bcl2) expression was examined.

3.5.1. Gene Expression of Caspase-3

The semi-q PCR of caspase-3 mRNA analysis showed that the *S. Montana* L. and *M. piperita* L. *var. citrata* aqueous extracts and essential oils exhibited an increase in the expression of the caspase-3 gene in the HepG2 cells as indicted by the amount of the mRNA. Caspase-3 expressions were significantly different ($P \leq 0.05$) from those of the untreated cells and the oils have wider bands of caspase-3 mRNA than the aqueous extracts (Figures 5 and 6).

3.5.2. Gene Expression of Bcl-2 Family

The *S. Montana* L. and *M. piperita* L. *var. citrata* aqueous and oil extracts' treated the HepG2 cells for forty-eight hours, and showed stronger pro-apoptotic Bax mRNA expressions than in the untreated cells. The Bcl-2 expressions of the *S. montana* L. and *M. piperita* L. *var. citrata* incubated cells were significantly ($P \leq 0.05$) reduced compared to the control cells. Greater alterations were noticed in the volatile oil treated cells, where it significantly decreased ($P \leq 0.05$) the mRNA expression of the pro-apoptotic (Bax) and the apoptosis regulator (Bcl-2) as shown in (Figures 5 and 6).

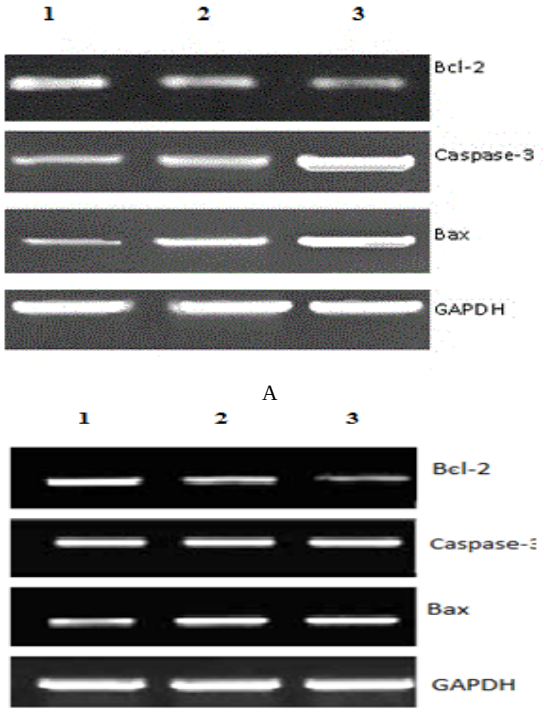

Figure 5. Effects of **(A)** *Satureja montana* L. **(B)** *Mentha piperita* L. *var. citrata* on the mRNA expression levels of caspase-3, Bcl-2 and Bax in HepG2 cells. GAPDH was used as an internal control. Where lane 1: untreated HepG2; lane 2: HepG2 treated with aqueous extract and lane 3: HepG2 treated with volatile oil.

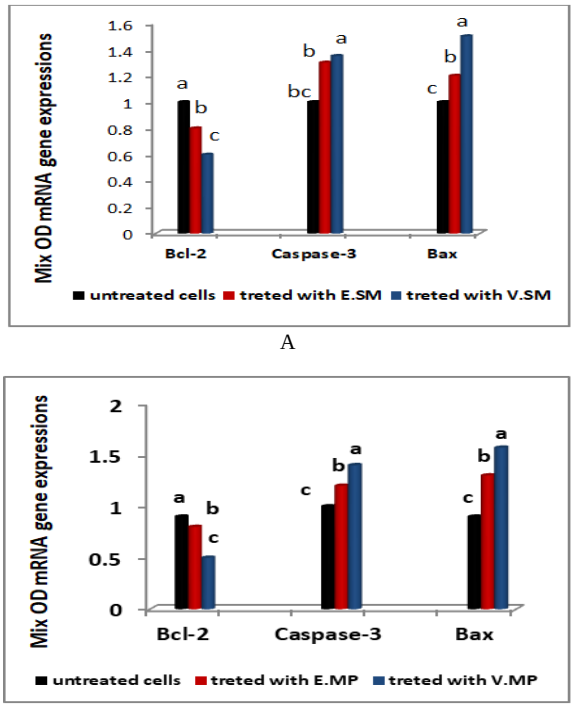

Figure 6. Effect of **(A)** *Satureja montana* L. and **(B)** *Mentha piperita* L. *var. citrata* extract and volatile on apoptotic gene expression level in HepG2 cell line4.The results illustrated are normalized to the level of GAPDH level and the data are the means of optical density for each gene divided by that for GAPDH.

4. Discussion

Most of aromatic and medicinal plants have compounds with antioxidant properties. Natural antioxidants are considered useful agents for diseases' prevention. Previous studies have shown that plant phenolic compounds display an antioxidant property because of their free radicals scavenging capacity (de Oliveira *et al*., 2011). The results of the current study revealed that the *S. montana* L. and *M. piperita* L. *var. citrata* extracts and oils contain a high phenolic content and showed high free radical-scavenging and reducing-power activities. These results are supported by the study of Pereira and Cardoso (2013), who confirmed that *M. piperita* L. plants are rich in phenolic compounds. Additionally, Hassanein *et al*. (2014) proved that *S. montana* cultivated in Egypt contains a high content of phenolic and flavonoid compounds that could be reliable for a remarkable radical scavenging and antioxidant properties. This promising efficiency suggests its potential role as an antioxidant agent, to improve the antioxidant condition and counteract the hazards of diseases related to oxidative stress.

Medicinal plants essential oils have a broad variety of biological activities including spasmolytic, hepato-protective, antiviral and anticarcinogenic effects (Lahlou, 2004). In recent years, many studies recognized the efficiency of essential oils and their chemical constituents as a source of novel bioactive natural products even against cancer (Piaru *et al*., 2012; Rasoanaivo *et al*., 2013; Zapata *et al*., 2014). Al-Okbi *et al*. (2015) revealed that *M. citrata* and *M. longifolia* own antioxidant and anticancer activities that could be referred to the occurrence of phytosterosl, phenolic compounds and specific volatile constituents. The present study's data of plant analysis revealed that the *S. montana* L. and *M. piperita* L. *var. citrata* essential oils possess stronger antioxidant properties than their extracts. This result is in agreement with Nemati *et al*. (2018) who reported that the *S. montana* L. essential oils have a prospect for reducing Fe^{+3} to Fe^{+2} and also Cu (II) to Cu (I), and possess a strong antioxidant activity.

The current study showed that the aromatic plants, *S. montana* L. and *M. piperita* L. *var. citrate*, affected the HepG2 cells viability. Here, the *in vitro* cytotoxic study reported that the extracts and oils significantly suppressed the growth of hepatocellular carcinoma human cells (HepG2) in a dose-dependent manner with the IC50 value of *S. montana* L. = 50 and 25 µg/mL and *M. piperita* L. *var. citrata* = 91.5 & 20.2 µg/mL, respectively. It can be concluded that the essential oils of *S. montana* L. and *M. piperita* L. *var. citrata.* are two and four times more cytotoxic than the aqueous extracts. This result is in accordance with Elgndi *et al*.(2017) who demonstrated that the essential oil and CO_2 extract of *S. montana* showed a high anti-proliferative activity (IC50 = 59.85–91.05 µg/mL) against HeLa cells line, where the essential oil is 1.5 times more cytotoxic than CO_2 extracts. Fitsiou *et al.* (2016) suggested that the essential oils of *S. thymbra* and *S. parnassica* exhibited a diverse anti-proliferative activity against A549 non-small cell lung adenocarcinoma and HepG2 and Hep3B liver hepatocellular carcinoma cells. Sharifi-Rad *et al.* (2015) reported that the *S. intermedia* essential oils showed a concentration-dependent decrease in the HepG2 and MCF-7 human cancer cell lines viabilities. Arunasree (2010) confirmed that the *S. montana* cytotoxicity may be attributed to the relatively high phenolic compounds concentrations, particularly carvacrol. They demonstrated the carvacrol antitumor effects on MCF-7, MDA-MB and suggested that it has a therapeutic potential significance in cancer treatment. At the same time, Hussain *et al.* (2010) reported that the *M. piperita* L. *var. citrata* essential oil showed prominent cytotoxicity against breast cancer (MCF-7) and prostate cancer (LNCaP) cell lines with IC50 ranging from (43.5 ± 2.1 to 95.7 ± 4.5 µg/mL). Abirami and Nirmala (2014) showed that the *M. piperita* ethanolic extract revealed a cytotoxicity against human laryngeal epidermoid carcinoma with IC50 = 94 µg/mL. In addition, Ogaly *et al.* (2018) reported that the *M. piperita* L. essential oil exhibited a protective property against hepatotoxicity.

Furthermore, the results illustrated that the mRNA expression of key apoptotic regulators Bax, Bcl-2 and caspase-3 was altered following the *S. montana* L. and *M. piperita* L. *var. citrata* cell lines treatment. This is supported by Abd El Tawab *et al.* (2014) who showed that the *S. montana* extract protects the rat testis against cyclophosphamide-induced damage via anti-oxidative and anti-apoptotic mechanisms. Yfanti *et al.* (2015) concluded that the *S. horvatii* showed anticancer activity through A549 cell apoptosis. In addition, Jain *et al.* (2011) showed that *M. piperita* induced significant dose and time-dependent anticarcinogenic activity, leading to cell cycle arrest and mitochondrial-mediated apoptosis, and upregulation of Bax, p53 and p21 genes in the treated cells. Bax expression is upregulated by the tumor suppressor protein (p53), and it was shown to be involved in the p53-mediated apoptosis. The results of the present work demonstrated that the *S. montana* L. and *M. piperita* L. *var. citrata* treatment increased Bax expression in the HepG2 cells.

Caspase-3-mediated proteolysis is an important element of the mitochondria mediated apoptotic process. Active caspase-3 comprises a heterodimer of 17- and 112-kDa subunits that in turn are resultant from a 32-kDa proenzyme, a marker for apoptotic cells. Active caspase-3 proteolytically cleaves and activates other caspases and relevant targets in the cytoplasm (Jain *et al*., 2011). The same signaling pathway was identified in the HepG2 cells and evaluated in the present study. Caspase-3 activation was previously confirmed to increase following the treatment of the cells with *Satureja montana* L. (Bhattacharjee & Chatterjee, 2013). Ferreira *et al.* (2014) found that the *M. piperita* L. essential oil fatal cytotoxicity is linked with the increased levels of intracellular ROS, mitochondrial fragmentation, and chromatin condensation, without loss of the plasma membrane integrity, indicative of an apoptotic process. The *S. montana* L. constituents interact with mitochondrial membranes and alter their permeability by opening transition pores decreasing the potential of the mitochondrial membrane (Abd El Tawab *et al.*, 2014). Therefore, *S. montana* L. induces structural changes and increases the mitochondrial membrane unsaturation. This mitochondrial alteration may affect the activity of pro-and anti-apoptotic proteins of the Bcl-2 family. It was previously demonstrated that *S. montana* L. contributes to the downregulation of the anti-apoptotic

molecule Bcl-2 expression, and blocks lipid peroxidation that inhibits the apoptosis induction (Cetkovic *et al*., 2007).

5. Conclusion

Based on earlier literature and the data of the present study, it was hypothesized that the *S. montana* L. and *M. piperita* L. *var. citrata* aqueous extracts or essential oils induce human hepatocellular carcinoma cell apoptosis. The signal transduction occurs via the caspase-3 signaling pathway. Extra investigation on this signaling pathway is necessary to clarify their function in tumor invasion and survival.

References

Abdel-Massih RM, Fares R, Bazzi S, El-Chami N and Baydoun E. 2010. The apoptotic and anti-proliferative activity of *Origanum majorana* extracts on human leukemic cell line. *Leukemia Res*, **34**:1052-1056.

Abd El Tawab AM, Shahin NN, and Abdel Mohsen MM. 2014. Protective effect of *Satureja montana* extract on cyclo-phosphamide-induced testicular injury in rats. *Chemico-Biol Interactions*, **224(5)**: 196-205.

Abirami SKG and Nirmala P. 2014. A comparative in vitro study of anticancer effect of *Mentha piperita*, *Ocimum Basilicum and Coleus aromaticus* against human laryngeal epidermoid carcinoma (HEP-2) cell lines. *J Med Plants Stud*, **2(1):** 6-9.

Adaszynska S M and Dzieciol M. 2017. Comparison of phenolic acids and flavonoids contents in various cultivars and parts of common lavender (*Lavandula angustifolia*) derived from Poland. *Nat Prod Res*, **31(21):** 2575–2580.

Al-Okbi S Y, H HM Fadel and Mohamed D A.2015. Phytochemical constituents, antioxidant and anticancer activity of *Mentha citrata* and *Mentha longifolia*. *Res J Pharmaceutic Biol Chem Sci*, **6(1):** 739-751

Arunasree K M. 2010. Anti-proliferative effects of carvacrol on a human metastatic breast cancer cell line, MDA-MB 231. *Phytomed*, **17(8–9):** 581–588, 2010.

Balogh J, Victor D, Asham E H, Burroughs S G, Boktour M, Saharia A, Li X, Ghobrial R M and Monsour Jr H P. 2016. Hepatocellular carcinoma: a review. *J Hepatocell Carcinoma*, **3:** 41–53.

Benabdallah A, Rahmoune C, Boumendjel M, Aissi O, Messaoud C. 2016. Total phenolic content and antioxidant activity of six wild *Mentha* species (*Lamiaceae*) from northeast of Algeria. *Asian Pac J Trop Biomed*, **6(9):** 760-766.

Bhattacharjee B and Chatterjee J. 2013. Identification of proapoptopic, anti-inflammatory, anti-proliferative, anti-invasive and anti-angiogenic targets of essential oils in cardamom by dual reverse virtual screening and binding pose analysis. *Asian Pac J Cancer Prevent*, **14:**3735–3742.

Berridge MV, Herst PM and Tan AS. 2005. Tetrazolium dyes as tools in cell biology: New insights into their cellular reduction. *Biotechnol Annu Rev*, **11:** 127-52.

Capuzzo A and Maffei ME. 2016. Molecular fingerprinting of peppermint (*Mentha piperita*) and some Mentha hybrids by sequencing and RFLP analysis of the 5S rRNA Non-Transcribed Spacer (NTS) region. *Plant Biosyst*, **150 (2):** 236-243.

Cetkovic GS, Canadanovic-Brunet J M, Djilas S M, Tumbas V T, Markov S L and Cvetkovic D D.2007. Antioxidant potential, lipid peroxidation inhibition and antimicrobial activities of *Satureja montana* L. subsp. *kitaibelii* extracts. *Int J Mol Sci*, **8(10):** 1013–102

Chinou I, Liolios C, Moreau D and Roussakis C. 2007. Cytotoxic activity of *Origanum dictamnus. Fitoterapia* , **78:** 342-344.

Darzi ES and Amirghofran Z. 2016. Dichloromethane fraction of *Melissa officinalis* induces apoptosis by activation of intrinsic and extrinsic pathways in human leukemia cell lines. *Immunopharmacol Immunotoxicol* , **35:**313-320.

de Oliveira TL, Soares A R, Ramos M E, Cardoso M G, Alves E and PiccoliH R. 2011. Antimicrobial activity of *Satureja montana* L. essential oil against *Clostridium perfringens* type A inoculated in mortadella-type sausages formulated with different levels of sodium nitrite. *Int J Food Microbiol*, **144(3):** 546–555.

Dunkic V, Bezic N, Vuko E and Cukrov D. 2010. Antiphytoviral Activity of *Satureja montana* L. ssp. Variegate (Host) P. W. Ball essential oil and phenol compounds on CMV and TMV. *Molecules*, **15:** 6713-6721.

Elgndi MA, Filip S, Pavlic B, Vladic J, Stanojkovic T, Zizak Z and Zekovic Z. 2017. Antioxidative and cytotoxic activity of essential oils and extracts of *Satureja montana* L., *Coriandrum sativum* L. and *Ocimum basilicum* L. obtained by supercritical fluid extraction. *J Supercrit Fluids*, **128:**128-137.

El-Serag HB and Rudolph KL. 2007. Hepatocellular carcinoma: Epidemiology and molecular carcinogenesis. *Gastroenterol*., **132:**2557–2576.

Ferreira P, Cardoso T, Ferreira F, Fernandes-Ferreira M, Piper P and Sousa J M. 2014. *Mentha piperita* essential oil induces apoptosis in yeast associated with both cytosolic and mitochondrial ROS-mediated damage. *FEMS Yeast Res*, **14(7):**1006–1014.

Fitsiou E, Anestopoulos I, Chlichlia K, Galanis A, Kourkoutas I, Panayiotidis MI and Pappa A. 2016. Antioxidant and antiproliferative properties of the essential oils of *Satureja thymbra* and *Satureja parnassica* and their major constituents. *Anticancer Res*. **36:**5757-5746.

Grosso C, Oliveira A C, Mainar AM, Urieta J S, Barroso J G and Palavra A M. 2009. Antioxidant activities of the supercritical and conventional *Satureja montana* extracts. *J Food Sci*, **74:** C713-C717.

Hadian J, Akramian M, Heydaria H, Mumivand H and Asghari B. 2012. Composition and in vitro antibacterial activity of essential oils from four *Satureja* species growing in Iran. *Nat Prod Res*, **26:**98–108.

Hassanein HD, Said-Al Ahl HAH and Abdelmohsen MM. 2014. Antioxidant polyphenolic constituents of *Satureja Montana* L. Growing in Egypt. *Int J Pharm Pharm Sci*, **6(4):**578-581

Hussain AI, Anwar F, Nigam PS, Ashraf M and Gilani AH.2010. Seasonal variation in content, chemical composition and antimicrobial and cytotoxic activities of essential oils from four *Mentha* species. *J Sci Food Agric*, **90 (11):** 1827–1836.

Jafari F, Ghavidel F and Zarshenas MM. 2016. A critical overview on the pharmacological and clinical aspects of popular *Satureja* species. *J Acupunct Meridian Stud*, **9:** 118-127.

Jain D, Pathak N, Khan S, Venkata RG, Bhargava A, Samarth R and Kumar M P. 2011. Evaluation of cytotoxicity and anticarcinogenic potential of *Mentha* leaf extracts. *Int J Toxicol* **30(2):** 225-236.

Lahlou M. 2004. Essential oils and fragrance compounds: bioactivity and mechanisms of action. *Flavour Fragr J*, **19:** 159-165.

Lerebour G, Lartaud P and Lacroix B. 2014. Essential oil of *Satureja montana* with a high geraniol content and use for treating

greasy skin and/or the associated aesthetic defects. US Patents No. US20160095812 A1.

Lin J M, Wei L H, X u W, Hong Z, Liu X and Peng J. 2011. Effect of *Hedyotis diffusa* wild extract on tumor angiogenesis. *Mol Med Rep*, **4**: 1283-1288.

Ling CQ, Yue XQ and Ling C. 2014.Three advantages of using traditional Chinese medicine to prevent and treat tumor. *J Integr Med*, **12(4):**331–5.

Menichini F, Tundis R, Bonesi M, Loizzo MR, Conforti F, Statti G, De Cindio B, Houghton PJ and Menichini F. 2009.The influence of fruit ripening on the phytochemical content and biological activity of *Capsicum chinense Jacq. cv Habanero*. *Food Chem*, **114**: 553–560

Nemati Z, Talebi E, Khosravinezhad M and Golkari H. 2018. Chemical composition and antioxidant activity of Iranian *Satureja montana*. *Sci Int*, **6 (1):** 39-43.

Ogaly A H, Eltablawy A N and Abd-Elsalam M R. 2018. Antifibrogenic influence of *Mentha piperita* l. essential oil against ccl_4-induced liver fibrosis in rats. *Oxid Med Cell Longev*, **2018**:1-15.

Oyaizu, M. 1986. Studies on product of browning reaction prepared from glucose amine. *Jpn. J. Nutr*, **44:** 307–315.

Pereira RO and Cardoso SM. 2013. Overview on *Mentha* and *Thymus* polyphenols. *Curr Anat Chem*, **9:**382-396.

Piaru S P, Mahmud R, Majid A M S A, Ismail S and Man C N. 2012. Chemical composition, antioxidant and cytotoxicity activities of the essential oils of *Myristica fragrans* and *Morinda citrifolia*. *J Sci Food Agric*, **92:**593–597.

Poon D, Anderson BO, Chen LT, Tanaka K, Lau WY, Van Cutsem E, Singh H, Chow WC, Ooi LL, Chow P, Khin MW and Koo WH. 2009. Management of hepatocellular carcinoma in Asia: Consensus Statement from the Asian Oncology Summit. *Lancet Oncol*, **10:**1111–1118.

Rasoanaivo P, Randriana R F, Maggi F, Nicolettid M, Quassinti L, Bramuccic M, Lupidic G, Petrellie D, Vitali L A, Papa F and Vittori S. 2013. Chemical composition and biological activities of the essential oil of *Athanasia brownie Hochr*. (Asteraceae) endemic to Madagascar. *Chem Biodivers*, **10(10):**1876–1886.

Raza A and Sood G K . 2014. Hepatocellular carcinoma review: Current treatment, and evidence-based medicine. *World J Gastroenterol*, **20 (15):** 4115–4127.

Rey M J, Pujol P, Callier P, Cavailles V, Freiss G, Maudelonde T and Brouillet P J. 2000. Semi quantitative reverse transcription-polymerase chain reaction to evaluate the expression patterns of genes involved in the oestrogen pathway. *J Mol Endocrinol*, **24:** 433–440.

Sharifi-Rad J, Sharifi-Rad M, Hoseini-Alfatemi M S, Iriti M, Sharifi-Rad M and Sharifi-Rad M. 2015. Composition, cytotoxic and antimicrobial activities of *Satureja intermedia* C.A.Mey essential oil. *Int J Mol Sci*, **16 (8):** 17812–17825.

Singleton VL, Orthofe R and Lamuela-Raventos RM.1999. Analysis of total phenols and other oxidation substrates and antioxidants by means of Folin Ciocalteu reagent. *Meth Enzymol*, **299:** 152–178.

Spiridon I, Colceru S, Anghel N, Teaca CA, Bodirlau R and Armatu A. 2011. Antioxidant capacity and total phenolic contents of oregano (*Origanum vulgare*), lavender (*Lavandula angustifolia*) and lemon balm (*Melissa offcinalis*) from Romania. *Nat Prod Res*, **25(17):** 1657–1661.

Sytar O, Hemmerich I, Zivcak M, Rauh C and Brestic M. 2018. Comparative analysis of bioactive phenolic compounds composition from 26 medicinal plants. *Saudi J Biol Sci*, **25(4):** 631-641.

Thaipong K, Boonprakob U and Crosby K. 2006. Comparison of ABTS, DPPH, FRAP and ORAC assays for estimating antioxidant activity from guava fruit extracts. *J Food Comp Anal*, **19:** 669–675.

Thomas M. 2009. Molecular targeted therapy for hepatocellular carcinoma. *J Gastroenterol*, **44 (Suppl 19):**136 –141.

Trifan A, Sava D, Bucur L, Vasincu A, Vasincu I, Aprotosoaie AC, Cioancă O and Miron A. 2015. Isolation, characterization and antioxidant activity of the crude polysaccharide from phyllophora pseudoceranoides. *Rev Med Chir Soc Med Nat Iasi*, **119(2):**603-609.

Vagionas K, Graikou K, Ngassapa O, Runyoro D and Chinou I. 2007. Composition and antimicrobial activity of the essential oils of three *Satureja* species growing in Tanzania. *Food Chem*, **103:** 319-324.

Yamaguchi T1, Takamura H, Matoba T and Terao J. 1998. HPLC method for evaluation of the free radical-scavenging activity of foods by using 1, 1-diphenyl-2-picrylhydrazyl. *Biosci Biotechnol Biochem*, **62 (6):**1201-4.

Yfanti P, Batistatou A, Manos G and Lekka M E. 2015. The aromatic plant *Satureja horvatii* ssp. macrophylla induces apoptosis and cell death to the A549 cancer cell line. *Am J Plant Sci*, **6:** 2092-2103.

Zapata B, Betancur L G, Duran C and Stashenko E. 2014. Cytotoxic activity of *Asteraceae* and *Verbenaceae* family essential oils. *J Essent Oil Res*, **6:**50–57.

Zhishen J, Mengcheng T and Jianming W. 1999.The determination of flavonoid contents in mulberry and their scavenging effects on superoxide radicals. *Food Chem*, **64:** 555–559.

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Isolation, Cloning, and Sequence Analysis of the Full-Length *RFT1* Gene from Malaysian Upland Rice (*Oryza sativa*, subsp. *Indica*, Cultivar Wai)

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Abstract

Rice Flowering locus T1 (*RFT1*) is the second rice florigen that promotes flowering during non-inductive long-day conditions. In this study, the full-length *RFT1* gene was isolated from the matured leaves of Malaysian upland rice (designated as Mu-*RFT1*), Wai cultivar of *indica* sub-species, and construct *pGMT:MuRFT1* was developed and successfully transformed into *E. coli* DH5α. The resulted gene sequence and phylogenetic relationships of ten other *indica*, two *japonica* rice and a single outgroup species were analyzed. The reverse transcription PCR and bioinformatics analyses demonstrated that the full-length Mu-*RFT1* shared 99 % nucleotide and 80 % amino acid identity with the other rice cultivars. Furthermore, the phylogenetic tree analysis revealed that the Mu-*RFT1* gene is closely related to Kemasin and shares common evolutionary ancestor characteristics including amino acid homology (99 %). This is the first study on the full-length *RFT1* gene isolation, cloning, and sequence analysis from upland rice, Malaysian cultivar. It also provides useful information on the phylogenetic relationship of the gene and its molecular evolution as well as designing a scientific breeding system for producing a novel variety of rice.

Keywords: Upland rice, *Rice Flowering locus T1* (*RFT1*) gene, Cloning, Phylogenetic tree, Sequence analysis.

1. Introduction

Rice (*Oryza sativa*) from the *Poaceae* family is the most important cereal crop and staple diet for many people worldwide. It is a model monocot plant widely used for genome organization and gene expression studies (Bajaj and Mohanty, 2005). The sub-species named *indica* comprises both the wetland and upland rice cultivars. The upland cultivars have numerous advantages including its potential as an alternative option for wetland rice and the possibility to be grown on dry-land or in rain-fed conditions. It is known to thrive on the surface, accumulated or phreatic or unbunded water supplies. The upland rice cultivar accounts for almost 80 % of rice cultivated worldwide, but contributes only to 12% of the global rice production due to the low yield and poor management practices (Din *et al.*, 2016). The *Oryza sativa* species is cultivated worldwide due to its diverse characteristics of flowering time. The florigen molecular and environmental signals such as photoperiod and temperature are the major factors that trigger floral induction processes (Liu *et al.*, 2018).

Scientific evidence from the molecular approach has indicated the existence of diverse evolutionary genes in rice that are uniquely acquired for promoting flowering. The flowering time in rice is basically determined by the expression of two essential yet highly conserved florigens including *Heading date 3a* (*Hd3a*) under short-day (SD) conditions and *Rice Flowering Locus T1 (RFT1*) under long-day (LD) conditions (Komiya *et al.*, 2009). The *RFT1* gene is the rice LD florigen precisely situated at 11.5 kb away from the *Hd3a* on chromosome six. The *RFT1* gene regulates heading date through a complex genetic network by translocation from the leaf to the shoot apical meristem (SAM). It generally interacts with the transcription factor *flowering locus D* (*FD*) using *RFT1*-GFP fusion from the leaves to the shoot apex. It reportedly functions as a mobile signal through a protein or mRNA to switch on the flowering process in rice cultivars (Itoh and Izawa, 2013; Komiya *et al.*, 2008). However, the *RFT1* gene has not been fully isolated from the upland rice variety of *indica* sub-species.

Additionally, understanding the gene function in rice cultivars growing under LD conditions is still unclear. Therefore, it is essential to further study the flowering system of upland rice, particularly the *RFT1* gene regulation. Arif *et al.* (2016) have only previously examined the integral RNA and *RFT1* protein isolation from the upland rice variety of *indica* sub-species but with no report on the full-length isolation. Therefore, this study presents the first report on the isolation, construction into

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cloning vector, and sequence analysis of full-length *RFT1* gene from the Malaysian upland rice. The study of the isolation of the full-length *RFT1* gene and its construction is of a significant interest, and will provide insights into the intrinsic gene function and its regulation mechanisms after plant transformation. Equally, the construction of a phylogram tree is important in the evolutionary studies of the gene. It is critical for inferring and clarifying the biological evolution and relationships between species.

2. Materials and Methods

2.1. Plant Material

Mature seeds of the Malaysian upland rice, cultivar Wai, were collected from Sibu, Sarawak Malaysia. The seeds were planted in pots based on the sandy soil to a compost ratio of 1:2, and were grown in the glasshouse of the Faculty of Science, Universiti Teknologi Malaysia, Johor Bahru, Malaysia.

2.2. RNA Isolation and cDNA Synthesis

The total mRNA was isolated from the 8-11 weeks old mature leaves using Trizol reagent (Sigma-Aldrich) based on the manufacturer's instruction. The DNase treatment was performed using DNase I Promega kit before the cDNA synthesis to eliminate any genomic DNA contamination. The quantity and purity of the mRNA were analyzed using Nanodrop^{TM} 1000 spectrophotometer at the absorbance ration of $A_{260}/A_{280.}$ Whereas, the mRNA quality was determined using agarose gel electrophoresis on 1 % (w/v) agarose in 1 X TAE buffer stained with SYBR safe (Invitrogen). Using the GoScript^{TM} reverse transcription system (Promega), the cDNA was synthesized by using 5 µg of poly $(A)^+$ mRNA, $Oligo(dT)_{18}$ primer and the reverse transcription enzyme according to the manufacturer's instructions. The cDNA was stored at -20 °C prior to subsequent experiment.

2.3. Reverse Transcription PCR Amplification of RFT1 Gene and Sequencing

The complete cDNA was used as a template for the reverse transcription PCR amplification of the full-length *RFT1* gene from the Malaysian upland rice (designated as Mu-*RFT1*) through gene-specific primers, as outlined in Table 1. The amplification was performed at 94°C for a four minute-pre-denaturation, followed by thirty cycles of denaturation for thirty seconds at 94°C. Annealing was implemented for forty seconds at 55°C, sequential extensions for one minute and twenty seconds at 72°C, and the final extension for five minutes at 72°C before cooling to 4°C. The amplicons were subjected to electrophoresis on 1% (w/v) agarose gel stained with SYBR safe (Invitrogen).

The gel bands of the PCR products were purified using Wizard SV gel and the PCR clean-up system (Promega) according to the manufacturer's instructions. Subsequently, the purified sample was sent to the First Base Sdn Bhd Malaysia for sequencing prior to the cloning experiment. The sequences were analyzed using bioinformatics tools (BLASTn and BLASTp) that are available in NCBI (Jin *et al.*, 2013) for identification and similarity respectively. The resulted nucleotide to amino acid translation was predicted using a tool provided by ExPASy as reported earlier by Jin *et al*. (2013) and Xu *et al*. (2012).

Table 1. Primers used for the *RFT1* gene amplification and molecular cloning

Primer name	Primer Sequence 5' - 3'
RFT1-EX1 (F)	TGGCTAGCTTAACCTTCCTG
RFT1-EX1 (R)	GTCTACCATCACCTGTAGGT
RFT1-EX4 (F)	CGGAGGGAGTATCTATTTTG
RFT1-EX4 (R)	CACACTTAAGAGCCTGCATG
RFT1-S1 (F)	GCTCGTGAAGGCAGGAGATA
RFT1-S1 (R)	TTTTTACATGGCGAGGCCGG
EX1-C (F)	TAAGCAGTCGACTGGCTAGCTTAACCTTCCTG
EX1-C (R)	GTCTACCATCACCTGTAGGTCTGCAGTGCTTA

NB: EX1-C is the cloning primer containing *SalI* and *PstI* restriction sites as underlined.

2.4. Construction of the RFT1 Gene and Bacterium Transformation

The cloning vector pGEM-T was constructed using the specific primers (EX1-C in Table 1) with *SalI* and *PstI* restriction sites designed to amplify the full-length Mu-*RFT1* gene. The pGEM-T plasmid (vector) and the purified gene fragment (insert) from the PCR product were double digested separately earlier using the *SalI* and *PstI* restriction enzymes (RE), and were ligated together using T4 ligase enzyme according to the manufacturer's instructions (Promega Catalog ID: 9PIM180). The recombinant vector was designated as *pGMT:MuRFT1* (Figure 1), and was transformed into the *Escherichia coli* DH5α strain by a heat-shock method (Sambrook and Russell, 2001). The recombinant bacteria were screened by a blue/white colony on ampicillin (50 µg mL^{-1}) selective plate supplemented with 120 µL of 20 mg/mL X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) and 40 µL of 100 mM IPTG (Isopropyl-β-D-1-thiogalactosidase). The white colony cells were selected for colony PCR and enzyme digestion analysis, and were then sequenced by First Base Sdn Bhd Malaysia for bioinformatics analysis of the *RFT1* gene insert.

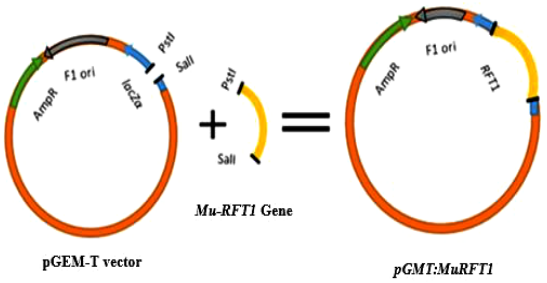

Figure 1. Ligation of pGEM-T vector and the *RFT1* gene at the *SalI* and *PstI* restriction sites designated as *pGMT:MuRFT1*.

2.5. Nucleotide Sequence and Molecular Evolution Analysis

The *RFT1* gene sequence from the Mu-*RFT1* cultivar was analyzed by BLASTn, and BLASTp algorithms (http://www.ncbi.nlm.nih.gov/) for identification and similarity searches. The nucleotides to amino acids translation was predicted using ExPASy (http://www.expasy.org/). Meanwhile, the deduced gene sequence from the Mu-*RFT1* cultivar with ten others from *indica* cultivars, two *japonica* cultivars and one outgroup species were selected for multiple sequence alignment. They included the *RFT1* gene from: Pokkali (BAO03221), Bleiyo (BAJ53916), Kemasin (BAO03216.1), Muha (BAJ53912), Basmati370 (BAH30236), Vandaran (BAO03225.1), Kasalath (BAH30234), Nona Bokra (BAX24675.1), Deng Pao Zhai (BAJ53911) and Tadukan (BAO03183.1) of *indica* sub-species, Nipponbare (BAB78480) and Dianyu 1 (BAO03202) from the *japonica* sub-species and *Oryza glumipatula* (BAH56284.1) as the outgroup gene extracted from the GenBank for the multi-sequence alignment. The sequence alignment was performed with ClustalX (Xu *et al.*, 2012). Subsequently, a phylogenetic tree of the sequences was constructed using Molecular Evolutionary Genetics Analysis (version 7.0, MEGA7) (Kumar *et al.*, 2016). The MEGA7 tree was generated by the maximum-likelihood method (Li *et al.*, 2017).

3. Results

3.1. Isolation and Characterization of the Mu-RFT1 Gene

Optimum concentration at 1,079.2±11.73 and purity 2.04±0.09 of the isolated mRNA were obtained by the spectrophotometric analysis, while the quality was validated on 1 % agarose which showed clear bands at 18S and 28S rRNA. The integrity bands analyses involving concentration, purity and rRNA bands determination indicated that the mRNA is contamination-free, and can be used for further experiment. Subsequently, the mRNA was used for downstream applications to amplify the *RFT1* gene from the Wai cultivar of Malaysia upland rice (Mu-*RFT1*).

The Mu-*RFT1* gene fragment with the size of 645 bp (Figure 2) was amplified using the EX1 and EX1-C primers. It was found that the deduced sequence from the methionine start codon to the stop codon was exactly 178 amino acids which corresponds to the findings of Ebana *et al.* (2011) and Ogiso-Tanaka *et al.* (2013). The authors reported that the *RFT1* gene has 178 amino acids or the equivalent to over 534 nucleotides. The Mu-*RFT1* gene sequence obtained in the present study has an additional nucleotide from the 5' and 3' sites (before the start codon and few after the stop codon) similar to the reports by Chen *et al.* (2004) and Zhu *et al.* (2017). This Mu-*RFT1* gene sequence residues were confirmed to be the *RFT1* gene after the BLAST analyses.

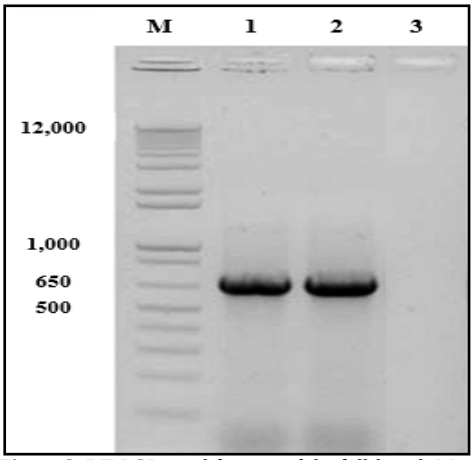

Figure 2. RT-PCR amplification of the full-length Mu-*RFT1* gene from the mature leaves of cultivar Wai. M; 1kb Plus DNA marker, Lane 1-2; Mu-*RFT1* gene, and Lane 3; negative control.

Figure 3 showed the sequencing result of the Mu-*RFT1* gene after the RT-PCR amplification. The outcome revealed a full-length isolation with the nucleotide composition of 99 %, and the amino acid residues showed an 80 % similarity to the *Oryza sativa* cultivars including Nona Bokra, Vandaran, and Kemasin. Further sequence analysis of the Mu-*RFT1* gene sequence obtained from the present study indicated some nucleotide diversity in comparison with the *RFT1* gene from the different candidate cultivars. This corresponds to the findings of Hagiwara *et al.* (2009) and Ogiso-Tanaka *et al.* (2013), which revealed changes in sixteen amino acids in the *RFT1* gene of *indica* cultivars. Previous gene analyses and transformations were conducted on *japonica* (Komiya *et al.*, 2009; Zhao *et al.*, 2015) or wetland *indica* (Ogiso-Tanaka *et al.*, 2013). Accordingly, the present study is the first report on the full-length *RFT1* gene isolation and characterization from the upland rice of *indica* sub-species.

```
atggcgggcagcggccgcgatgatccgctggtggtgggccgcattgtgggcgatgtgctg
 M  A  G  S  G  R  D  D  P  L  V  V  G  R  I  V  G  D  V  L
gatccgtttgtgcgcattaccaacctgagcgcgagctatggcgcgcgcattgtgagcaac
 D  P  F  V  R  I  T  N  L  S  A  S  Y  G  A  R  I  V  S  N
ggctgcgaactgaaaccgagcatggtgacccagcagccgcgcgtggtggtgggcggcaac
 G  C  E  L  K  P  S  M  V  T  Q  Q  P  R  V  V  V  G  G  N
gatatgcgcacctttttataccctggtgcgcattattagctggatgcagcgcccgaccaac
 D  M  R  T  F  Y  T  L  V  R  I  I  S  W  M  Q  R  P  T  N
ccgaacctgcgcgaatatctgcattggctggtgaccgatattccgggcaccaccggcgcg
 P  N  L  R  E  Y  L  H  W  L  V  T  D  I  P  G  T  T  G  A
acctttggccagaaagtgatgtgctatcgcagctatctgattacctttattattcatcgc
 T  F  G  Q  K  V  M  C  Y  R  S  Y  L  I  T  F  I  I  H  R
ctgaactatcataaaacccgcctgattatttttttttacctggatgcatgtgaactttgtg
 L  N  Y  H  K  T  R  L  I  I  F  F  T  W  M  H  V  N  F  V
tattttcatctgaccaaagattttgcggaactgtataacctgggcagcccggtggcggcg
 Y  F  H  L  T  K  D  F  A  E  L  Y  N  L  G  S  P  V  A  A
gtgtattttaactgccagcgcgaagcgggcagcggcggccgccgcgtgtatccgtag
 V  Y  F  N  C  Q  R  E  A  G  S  G  G  R  R  V  Y  P  *
```

Figure 3. Nucleotide and the deduced amino acid sequence of the Mu-*RFT1* gene. Start codon is shown in bold and stop codon is indicated by an asterisk and bold font. Both nucleotide and amino acid equivalent was predicted using the ExPASy tool.

3.2. RFT1 Gene Construction into the Cloning Vector

The construct *pGMT:MuRFT1* was successfully transformed into the *E. coli* DH5α. The positive transformants screening involves growing the cells on ampicillin (50 µg mL^{-1}) plates which were differentiated by white and blue colonies. From the plate, multiple white and light blue cell colonies were obtained. Thus, only the

dense white colonies were selected and used for subsequent experiments in this study. Furthermore, the success of gene cloning was examined by colony PCR analysis of the constructed vector (*pGMT:MuRFT1*) as shown in Figure 4. The results revealed full-length Mu-*RFT1* gene isolation from the recombinant vector which demonstrates the efficiency of the amplification process.

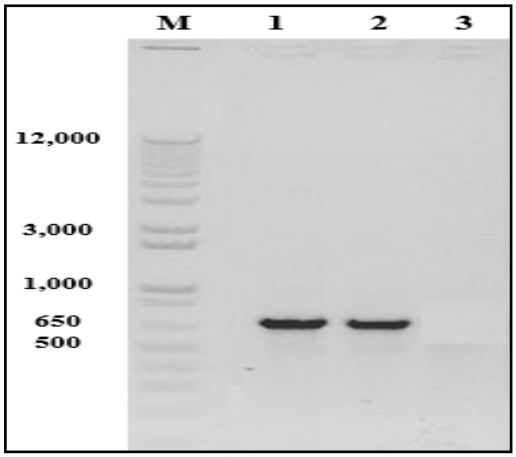

Figure 4. Colony PCR of the recombinant cells using EX1-C primers: M; 1kb Plus DNA marker, Lane 1, 2; Mu-*RFT1* gene from the *pGMT:MuRFT1* construct and Lane 3; negative control.

Double digestion of the *pGMT:MuRFT1* construct isolated from the recombinant cells using the *SalI* and *PstI* enzymes revealed two separate bands on the gel which depicts the vector and insert as shown in Figure 5. Therefore, the digestion analysis further confirmed the presence of the insert gene in the constructed vector. The *RFT1* gene band from the construct was excised, purified, and sequenced. The nucleotide and amino acid sequences were analyzed for further confirmation. The results revealed a 99 % nucleotide similarity to Keiboba, Kemasin, Nona Bokra, Tadukan and Vandaran (with 99 % query cover and an E-value of 0.0).

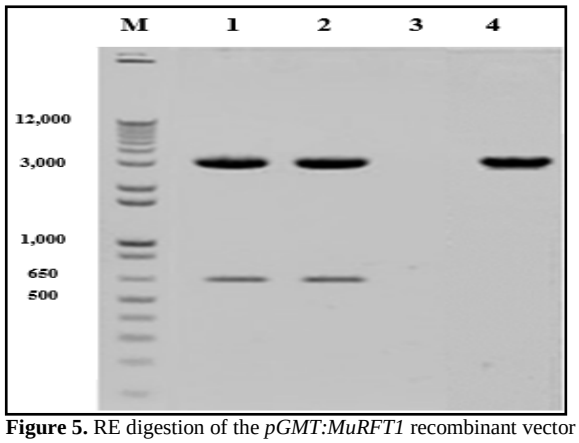

Figure 5. RE digestion of the *pGMT:MuRFT1* recombinant vector using *SalI* and *PstI* enzymes. M; 1kb plus marker, Lane 1-2; vector (at up 300 bp) and insert (at down 645 bp) separation, Lane 3; negative control and Lane 4; control vector.

3.3. Molecular Evolutionary Relationship Analysis of the RFT1 Gene

The multiple sequence alignment of the *RFT1* gene was examined through ClustalX tool. Figure 6 shows the *RFT1* amino acid sequence with high conservation at more than 90 % of the coding sequence. On the amino acids 31, 146 and 160, Basmati370 (*indica*), Nipponbare (*japonica*) and Dianyu1 (*japonica*) showed a unique amino acid (1) similarity which indicated their close evolutionary relationship, as denoted in black. A phylogenetic tree was constructed through the maximum likelihood method by the MEGA7 software. This was to identify and investigate the evolutionary relationships between the Mu-*RFT1* gene sequences from diverse *Oryza sativa* cultivars and a single *Oryza glumipatula* as the outgroup. The relationship of all the rice cultivars is represented in the phylogram in Figure 7.

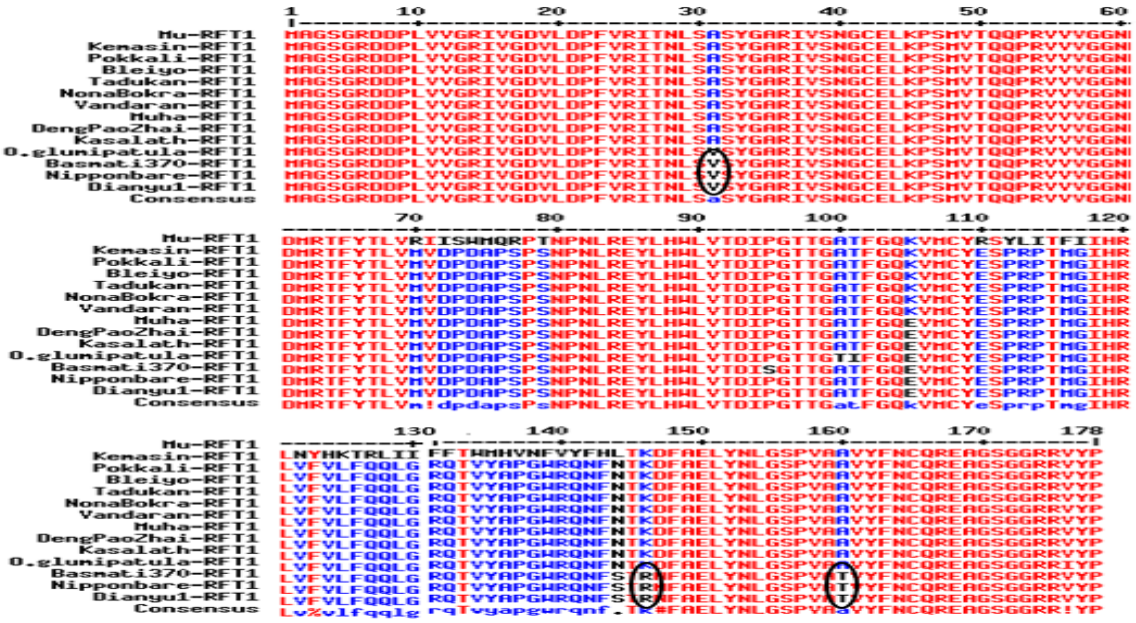

Figure 6. Multisequence alignment of the deduced Mu-*RFT1* gene amino acids obtained from the present study with that of the other rice cultivars using MEGA7. The other cultivars include Kemasin (BAO03216.1), Pokkali (BAO03221), Bleiyo (BAJ53916), Tadukan (BAO03183.1), Nona Bokra (BAX24675.1), Vandaran (BAO03225.1), Muha (BAJ53912), Deng Pao Zhai (BAJ53911), Kasalath (BAH30234), Basmati370 (BAH30236), Nipponbare (BAB78480), Dianyu 1 (BAO03202) and *Oryza glumipatula* (BAH56284.1).

In the phylogenetic tree, the cultivars were classified into two main groups. The first group comprises all the *Oryza sativa* sub-species and their cultivars, while the second group only contained *Oryza glumipatula*. The *Oryza sativa* collection was further categorized into two more sub-groups and clustered. The phylogenetic tree revealed that the Mu-*RFT1* gene was clustered with Kemasin, and are closely related to the *indica* cultivars including Pokkali, Bleiyo, Muha, Deng Pao Zhai, Kasalath, Nona Bokra and Vandaran (Figure 7). Of all the *Oryza sativa* cultivars, Tadukan exists independently in one sub-clade. The *japonica* cultivars; Nipponbare and Dianyu1 existed in the same cluster but formed a sub-clade with Basmati370 (Figure 7).

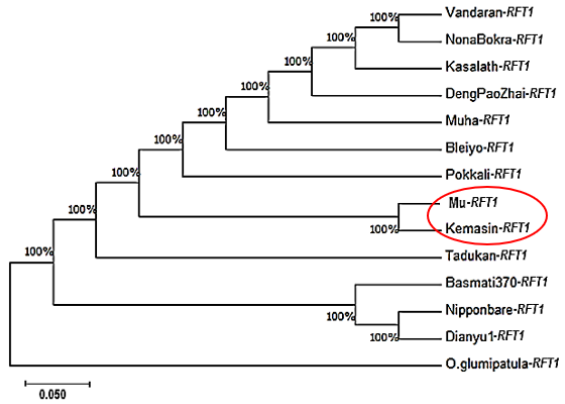

Figure 7. A phylogenetic tree derived from multiple sequence alignment using MEGA7; illustrating the relationships between the Mu-*RFT1* and corresponding genes from other cultivars including Vandaran (BAO03225.1), Nona Bokra (BAX24675.1), Kasalath (BAH30234), Deng Pao Zhai (BAJ53911), Muha (BAJ 53912), Bleiyo (BAJ53916), Pokkali (BAO03221), Kemasin (BAO03216.1), Tadukan (BAO03183.1), Basmati370 (BAH 30236), Nipponbare (BAB78480), Dianyu 1 (BAO03202) and *Oryza glumipatula* (BAH56284.1).

4. Discussion

4.1. RFT1 Florigen and its Cloning Construction

The most important prerequisite in the nucleic acid downstream application is the isolation of high-quality RNA (Zhang *et al.*, 2012). This step serves as a key evidential factor for further analyses through cDNA synthesis, reverse transcription PCR, microarray or cDNA library construction. As reported by Portillo *et al.* (2006), Trizol is the best reagent for RNA isolation from plant tissues compared to other methods as also confirmed by this study. Therefore, this is evidence for producing pure and high concentrated RNA from the matured leaves of the Malaysian upland rice, cultivar Wai of *indica* sub-species and subsequence *RFT1* gene downstream amplification. Research on the *RFT1* gene has recently attracted attention as it plays a crucial reproductive role by promoting flowering in rice at LD conditions, although the rice is an SD plant (Komiya *et al.*, 2008; Komiya *et al.*, 2009).

The *RFT1* gene is the second rice florigen and has been hypothesized as a hormone-like molecule for promoting flowering processes under LD conditions. This gene which is located on rice chromosome 6, is an ortholog to *FT* in *Arabidopsis thaliana* (Komiya *et al*., 2009). The gene is normally produced in the leaves, but interacts with the transcriptional factor *flowering locus D* (*FD*). Furthermore, it acts as a mobile signal in SAM buds and growing tips to control floral transition mainly by dual regulation of the *Early heading date 1* (*Ehd1*) (Komiya *et al.*, 2009; Sun *et al.*, 2016; Zhao *et al.*, 2015). Under LD conditions, rice flowering is regulated by the *Ehd1* promoter which is the B-type response regulator of *FT*-like genes that also up-regulates the positive regulator *RFT1*. This can also act as a constitutive activator and flowering inducer. The *Ehd1* and *Hd1* (*heading date 1* that promotes and activates heading) concurrently promote floral transition preferentially under SD and are antagonistic at LD conditions. This revealed the double function of *Ehd1* under both conditions (Chen *et al.*, 2018).

Evidence indicates that overexpression of the *RFT1* gene with the vascular-specific or constitutive promoter results in an early-flowering phenotype under LD conditions. However, its suppression by RNA-interference (RNAi) delays occurrence of the flowering (Komiya *et al.*, 2009). Moreover, the nucleotide diversity of the *indica* rice *RFT1* genes are closely related to the cultivar's genotype and regional distribution (Zhao *et al.*, 2015). This suggest that the functional constraint was relaxed in the *RFT1* gene after its duplication, which also demonstrates the haplotype diversity of the *RFT1* gene in the cultivated rice. The *RFT1* haplotype number is larger in the entire gene region, but smaller in the coding region (Ogiso-Tanaka *et al.*, 2013). The gene plays a crucial role in the reproductive cycle of rice cultivars in the LD environment. Hence, full-length isolation of Mu-*RFT1* gene and cloning are crucial for future genetic transformation.

The *pGMT:MuRFT1* was successfully constructed and introduced into the *E. coli* DH5α bacterium cell. The hand-pick screening of the transformed cells indicated the victory of the cloning and transformation analysis. Furthermore, the gene construction, recombinant DNA transformation and screening of transformants are important in molecular biology (Padmanabhan *et al.*, 2011). The presence of ampicillin plus X-gal and IPTG in the growth selective medium really simplifies the process of identifying the positive cloned from the negative transformants. In addition, the gene amplification via colony PCR and RE digestion were performed. The two separate analysis and victory obtained further demonstrated the success of the construction analysis. The colony PCR simply determined the presence of insert in the recombinant cells as well as sensitivity of the primers used for amplification, whereas the RE digestion excised the insert from the recombinant vector. The colony PCR screening techniques are cost-effective, fast, simple, and require no additional steps compared to sonication, mechanical high-speed cell disruption or the use of toxic chemicals (Mirhendi *et al.*, 2007). Additionally, restriction enzyme digestion is considered as the most reliable and accurate technique for verifying the plasmid constructs in the gene isolation and cloning experiments (Glover, 2013).

4.2. The Phylogeny Relationship of the RFT1 Genes

Construction of the phylogram tree is important in evolutionary studies, vital for inferring and clarifying the biological evolution and relationships between species or cultivars of the same origin. The relationship between thirteen amino acid sequences of the *RFT1* genes in the datasets obtained from Wai cultivar in this study was

analyzed with all gaps and missing data elimination. The phylogram shows the consensus phylogenetic relation amongst the thirteen *Oryza sativa* cultivars belonging to two sub-species and the *Oryza glumipatula* as an outgroup. This is based on a consistency index (CI) of 1 and retention index (RI) of 1, which indicated a steadily complete phylogenic character without homoplasy (Nei and Kumar, 2000; Arif *et al.*, 2016). The analysis confirmed the Mu-*RFT1* cultivar originates from the *indica* sub-species. However, the Basmati370 *RFT1* gene indicated some differences in nucleotides and amino acids compared to the other *indica* cultivars. This finding corresponds with the previous findings of Kovach *et al.* (2009) which revealed that Basmati370 shares a close evolutionary relationship with the *japonica* varieties based on its fragrant characteristic.

The discoveries presented in this study provide novel insights into the relationship between the aligned cultivars. Furthermore, the phylogeny analysis illustrates the evolutionary relationship between the rice cultivars and the outgroup species. Moreover, it inferred classification of the diverse *indica* cultivars in the study. This finding is supported by the distinct phenotypic characteristics of rice cultivars. For example, in terms of appearance and size, *indica* varieties possess slender and long grains, while *japonica* has stumpy and short grains. Historically, *indica* cultivars originated from India, Thailand and Indonesia, whereas *japonica* cultivars are from China, Japan, Laos, Taiwan and Vietnam. Therefore, there is a high degree of similarity in the gene sequences of the verified cultivars as was discovered in the present study. This corresponds with the suggestion by Hagiwara *et al.* (2009) who revealed that the *RFT1* gene had a high variability in Asia based on its phylogenetic relations.

5. Conclusion

Isolation of the qualified RNA and full-length Mu-*RFT1* gene were performed in the current study. The gene was subsequently constructed into the cloning vector pGEM-T and transformed into the *E. coli* DH5α strain, followed by characterization and molecular evolutionary relationship analysis of the gene sequences. The phylogenetic tree and amino acid sequence analyses demonstrated that the Mu-*RFT1* gene was closely related to Kemasin as the *indica* rice cultivar which grows in LD environments. Similarly, the multiple sequence alignment of the *RFT1* proteins from the *indica* and *japonica* cultivars and the out-group species showed that the florigen is well-conserved in the genus *Oryza*. This is a milestone in the molecular analysis, gene construction, and phylogeny characterization of the *indica* sub-species of upland rice. Consequently, a better understanding of the *RFT1* gene has been scrutinized to provide a better understanding of flower development at the molecular level. Furthermore, the data generated from this study provides an important understanding of the rice flowering system under LD conditions. The gene examined in this study is a potential target for genetic transformation and is required for producing a transgenic rice variety with improved reproductive phases, particularly, the flowering development. However, it is noteworthy to state that this part of the current research only presented data on the gene isolation, sequencing, phylogenic relation, and cloning vector construction of the *RFT1* gene in an upland rice, Malaysian cultivar. Therefore, this will be followed by a study on the expression construct development, plant genetic transformation of the gene to test its potentiality toward enhancing early flowering in rice. The novel discovery in this study is an indication of the prospects of expression construct, genetic transformation, and efforts to improve the flowering process of the upland rice cultivars.

Disclosure Statement

No competing financial interests or any conflict exist between the authors.

References

Arif, S. M., Dogara, AM, Ma, NL, Omar, MSS, Parvizpour, S and Rahmat, Z. 2016. Molecular phylogeny and structure prediction of rice *RFT1* protein. *J Teknologi*, **78**: 115-120.

Bajaj, S and Mohanty, A. 2005. Recent advances in rice biotechnology—towards genetically superior transgenic rice. *Plant Biotech J.*, **3**: 275-307.

Chen, JY, Zhang, HW, Zhang, HL, Ying, JZ, Ma, LY and Zhuang, JY. 2018. Natural variation at *qHd1* affects heading date acceleration at high temperatures with pleiotropism for yield traits in rice. *BMC Plant Biol.*, **18**: 112.

Chen, XH, Liu, QQ, Wu, HK, Wang, ZY and Gu, MH. 2004. cDNA cloning and sequence analysis of rice *sbe1* and *sbe3* genes. *Rice Sci.*, **11**: 81-85.

Din, ARJM, Ahmad, FI, Wagiran, A, Samad, AA, Rahmat, Z and Sarmidi, MR. 2016. Improvement of efficient *in vitro* regeneration potential of mature callus induced from Malaysian upland rice seed (*Oryza sativa cv.* Panderas). *Saudi J Biol Sci.*, **23**: S69-S77.

Ebana, K, Shibaya, T, Wu, J, Matsubara, K, Kanamori, H, Yamane, H, Yamanouchi, U, Mizubayashi, T, Kono, I and Shomura, A. 2011. Uncovering of major genetic factors generating naturally occurring variation in heading date among Asian rice cultivars. *Theor and Applied Gen.*, **122**: 1199-1210.

Glover, DM. 2013. **Genetic Engineering Cloning DNA**, Springer Science & Business Media.

Hagiwara, WE, Uwatoko, N, Sasaki, A, Matsubara, K, Nagano, H, Onishi, K and Sano, Y. 2009. Diversification in flowering time due to tandem *FT* - like gene duplication, generating novel Mendelian factors in wild and cultivated rice. *Mol Eco.*, **18**: 1537-1549.

Itoh, H and Izawa, T. 2013. The coincidence of critical day length recognition for florigen gene expression and floral transition under long-day conditions in rice. *Mol Plant*, **6**: 635-649.

Jin, Q, Yao, Y, Cai, Y. and Lin, Y. 2013. Molecular cloning and sequence analysis of a phenylalanine ammonia-lyase gene from Dendrobium. *PloS one*, **8**: e62352.

Komiya, R, Ikegami, A, Tamaki, S, Yokoi, S and Shimamoto, K. 2008. *Hd3a* and *RFT1* are essential for flowering in rice. *Development*, **135**: 767-774.

Komiya, R, Yokoi, S and Shimamoto, K. 2009. A gene network for long-day flowering activates *RFT1* encoding a mobile flowering signal in rice. *Development*, **136**: 3443-3450.

Kovach, MJ, Calingacion, MN, Fitzgerald, MA and Mccouch, SR. 2009. The origin and evolution of fragrance in rice (*Oryza sativa* L.). *Proceedings of the National Academy of Sci.*, **106**: 14444-14449.

Kumar, S, Stecher, G and Tamura, K. 2016. MEGA7: Molecular Evolutionary Genetics Analysis version 7.0 for bigger datasets. *Mol Bio and Evo.*, **33**: 1870-1874.

Li, L, Ye, T, Xu, J, Xie, C, Gao, X, Chen, R, Zhu, J and Xu, Z. 2017. Molecular characterization and function analysis of the rice *OsDUF946* family. *Biotech Biotech Equip.*, **31**: 477-485.

Liu, C, Qu, X, Zhou, Y, Song, G, Abiri, N, Xiao, Y, Liang, F, Jiang, D, Hu, Z and Yang, D. 2018. *OsPRR37* confers an expanded regulation of the diurnal rhythms of the transcriptome and photoperiodic flowering pathways in rice. *Plant, Cell Env.*, **41**: 630-645.

Mirhendi, H, Diba, K, Rezaei, A, Jalalizand, N, Hosseinpur, L and Khodadadi, H. 2007. Colony PCR is a rapid and sensitive method for DNA amplification in yeasts. *Iranian J Public Heal.*, **36**: 40-44.

Nei, M and Kumar, S. 2000. **Molecular Evolution and Phylogenetics**, Oxford university press.

Ogiso-Tanaka, E, Matsubara, K, Yamamoto, SI, Nonoue, Y, Wu, J, Fujisawa, H, Ishikubo, H, Tanaka, T, Ando, T and Matsumoto, T. 2013. Natural variation of the *Rice Flowering Locus T 1* contributes to flowering time divergence in rice. *PLoS One*, **8**: e75959.

Padmanabhan, S, Mandi, N and Banerjee, S. 2011. **Screening of Bacterial Recombinants: Strategies and Preventing False Positives**, INTECH Open Access Publisher.

Portillo, M, Fenoll, C and Escobar, C. 2006. Evaluation of different RNA extraction methods for small quantities of plant tissue: Combined effects of reagent type and homogenization procedure on RNA quality - integrity and yield. *Phys Plant*, **128**: 1-7.

Sambrook, J and Russell, DW. 2001. **Molecular Cloning: a Laboratory Manual**, 3rd ed. Coldspring-Harbour Laboratory Press, UK.

Sun, X, Zhang, Z, Wu, J, Cui, X, Feng, D, Wang, K, Xu, M, Zhou, L, Han, X and Gu, X. 2016. The *Oryza sativa* Regulator *HDR1* Associates with the Kinase *OsK4* to Control Photoperiodic Flowering. *PLoS Gen.*, **12**: e1005927.

Xu, F, Deng, G, Cheng, S, Zhang, W, Huang, X, Li, L, Cheng, H, Rong, X and Li, J. 2012. Molecular cloning, characterization and expression of the phenylalanine ammonia-lyase gene from *Juglans regia*. *Molecules*, **17**: 7810-7823.

Zhang, Y, Qin, Y, Guo, L, Zhou, Z, Liang, Z, Zhang, C and Guo, H. 2012. Isolation of high quality RNA from *Polyporus umbellatus* (Pers.) Fries. *Elect J Biotech.*, **15**: 10-10.

Zhao, J, Chen, H, Ren, D, Tang, H, Qiu, R, Feng, J, Long, Y, Niu, B, Chen, D and Zhong, T. 2015. Genetic interactions between diverged alleles of *Early heading date 1* (*Ehd1*) and *Heading date 3a* (*Hd3a*)/*Rice Flowering Locus T1* (*RFT1*) control differential heading and contribute to regional adaptation in rice (*Oryza sativa*). *New Phyt.*, **208**: 936-948.

Zhu, YJ, Fan, YY, Wang, K, Huang, DR, Liu, WZ, Ying, JZ and Zhuang, JY. 2017. *Rice Flowering Locus T 1* plays an important role in heading date influencing yield traits in rice. *Sci. Rep.*, **7**: 1-11.

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Evaluation of Repellent Potential of Some Botanical Products against Cowpea Weevil, *Callosobruchus maculatus* (Fabricius) (Coleoptera: Bruchidae)

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Abstract

The powder and crude extracts from five plant species, namely garlic (*Allium sativum*), manjack or drum tree (*Cordia millenii*), African or calabash nutmeg (*Monodora myristica*), negro-pepper (*Xylopia aethiopica*), and ginger (*Zingiber officinale*) were tested for their repellent activity against *Callosobruchus maculatus* (F.) adults through the cup bioassay technique and filter paper repellency method in the laboratory. *C. millenii* was the most superior repellent against *C. maculatus.* Results indicated that the repellent efficacy of the botanical materials followed this trend: *C. millenii* > *Z. officinale* > *A. sativum* > *X. aethiopica* > *M. myristica.* The former two plants generated a repellency of 66.0% and 65.4%, respectively; Class IV repellency (60.1-80.0%). Whereas, *A. sativum, X. aethiopica* and *M. myristica* caused a repellency of 50.3, 49.9 and 45.5%, respectively; Class III repellency (40.1-60.0%). The repellent effect on *C. maculatus* at the application doses of the powders of the selected plant species were statistically significant. There was also a significant difference in repellency due to various concentrations of the extracts of selected plant species with high correlation coefficients and positive significance compared to the powder treatment. Thus, % repellency increased according to doses and concentrations of the tested botanical products and due to the increase in the exposure period.

Keywords: Repellency, *Callosobruchus maculatus*, Botanical products, Bioassay and repellents.

1. Introduction

Callosobruchus maculatus (Fab.), a bruchid coleopteran commonly known as cowpea weevil, is a severe insect pest of stored grains including cowpea seeds (*Vigna unguiculata* (L.) in Sub-Sahara Africa (Al-Moajel and Al-Fuhaid, 2003) where it is responsible for up to a 100 % loss/damage of the seeds in storage and weight loss of about 60 % (Gbaye *et al.,* 2011). Caswell (1981) reported a loss of about 50 % of stored cowpea seeds, three-four months postharvest in Northern Nigeria, and 60 % cowpea seed loss to cowpea weevil in Northern Ghana (Tanzubil, 1991). The damage of this magnitude is incredibly high, and demonstrates the destructive nature of the pest which can threaten food security at both household and national levels. This is a major agricultural problem for farmers in developing countries (Ito and Ighere, 2017a).

Cowpea seeds damaged by bruchids are unfit for consumption, sales and planting because of perforation, weight reduction, overall unacceptability in markets and the impaired germination of the seeds (Ito and Ighere, 2017b; Uyi and Obi, 2017). Consequently, farmers are compelled to sell their products early after harvest when prices are still low partly because of anticipated losses of the grain in storage (Ito and Ighere, 2017a). This is a major and worrisome agricultural problem facing farmers in developing countries. Cowpea is an important source of dietary protein in tropical and subtropical regions of the world especially where availability and consumption of animal protein is low (Ofuya, 1991). The protection of cowpea against *C. maculatus* infestation and damage is necessary because of its economic importance as revenue source and constituent articles of diet.

Several control measures have been adopted over the years to curtail the menace of storage insect pests (Boeke *et al.,* 2004; Ogbonna *et al.,* 2016; Ito and Ighere, 2017a; Uyi and Obi, 2017; Ito and Utebor, 2018). Synthetic insecticides such as Dichlorodiphenyltrichloroethane (DDT) and Lindane (Srivastava and Pant, 1998) as well as botanical products including ashes, powders, and oil have been applied traditionally in the control of insects. The use of plant-derived insecticides was abandoned in favour of synthetic insecticides discovered in 1940s. However, the human-made insecticides became unpopular and wasteful (Ewete *et al*., 1996) owing to their prohibitive cost and technical difficulty of application by the majority of African peasant farmers. Besides, these insecticides are inimical to the environment because of toxic residues in

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food, pest resistance, and their negative impact on non-target beneficial insects (Cherry *et al*., 2005). These deficiencies made farmers shift away from the reliance on synthetic chemicals towards the use of plant materials to protect plants and stored-food products because they are biodegradable, environmentally-safe, and can delay pest resistance (Ito and Ighere, 2017b).

This study is carried out to determine the repellent potential of the powder and crude extracts from five spicy plant species relative to one another at various doses and concentrations against cowpea weevil *C. maculatus*.

2. Materials and Methods

2.1. Plant Materials

The plant species used for the study included garlic (*Allium sativum;* Family: Amaryllidaceae), manjack (*Cordia millenii*; Family: Boraginaceae), nutmeg (*Monodora myristica*; Family: Myristicaceae), negropepper (*Xylopia aethiopica*; Family: Annonaceae) and ginger (*Zingiber officinale;* Family Zingiberaceae). The plant parts used were: bulb, seed, seed, fruit and rhizome, respectively. The plant materials were obtained from a local market in Abraka, Delta State, Nigeria, and were processed into powder and crude extracts.

2.2. Preparation of Plant Powders

The plant materials were cut into smaller fragments and dried under the sun for seven days (Ito and Ighere, 2017a) and were later maintained in an oven at 60 ^{0}C for five-ten minutes to ensure that the plant materials were dried to constant weight. This treatment was to make sure that the extracts derived from them were devoid of water. The dry plants' materials were each pulverized with the aid of a Binatone electric blender (Model: BLG-400) and sieved through a fine mesh to obtain the powder which was stored in separate labelled airtight bottles to avoid loss of potency (Ito and Ukpohwo, 2018) until required for the repellency test (Okonkwo and Okoye, 1996).

2.3. Preparation of Crude Extracts

The extracts of each plant species were prepared by weighing out 25.0, 37.5 and 50.0 g from the milled powder into three separate 1000ml capacity glass jars and adding 500 mL of 95% ethanol (solvent) into the jars. These preparations corresponded to the concentrations of 50.0, 75.0 and 100.0 mg^{-ml} respectively. The jars containing the extract were shaken regularly and stirred vigorously with a glass rod for a period of three days before filtering using a filter paper (Whatman no. 1). The filtrates were concentrated by a slight application of heat in a water-bath system to evaporate the solvent. Viscous extracts were obtained and stored in airtight bottles in a refrigerator maintained at 5-10 ^{o}C until ready for use.

2.4. Culturing of Callosobruchus maculatus

The cowpea weevil, *C. maculatus*, was reared on cowpea grain by the method of Ito and Ighere (2017a) with modification in plastic containers (1.0 kg). Seeds of Cowpea (*Vigna unguiculata*) that were apparently un-infested with *C. maculatus* and those heavily infested were obtained from traders at a local market in Abraka, Delta State. Batches of 500g un-infested cowpea seeds were placed in each of the seven plastic containers used for the culture. Adult cowpea weevils were picked from the infested cowpea seeds to establish the stock from which batches of 100 unsexed weevils were taken and placed in each culture container. The containers were then covered with polythene nets fastened with rubber bands and were kept for seven days for mating and oviposition to occur. The parent weevils were then removed, and the culture was kept for 65-70 days (Ito and Ighere, 2017a). The adult first filial generation (F_1) weevils that emerged were taken and used for the study.

2.5. Repellency Bioassay with Plants' Powder

Repellency bioassay of the plants' powders against *C. maculatus* was carried out using the cup bioassay techniques of Kumar *et al.* (2004). The cup is a perforated cylinder (10 cm X 7 cm) made of thin aluminum sheet and covered at one end with a lid bearing pores through which weevil could move into a plastic container on which the cup was suspended. The container with the cup was placed in a trough where the weevils that emigrated through the pores in the cup were collected.

The plants' powders were tested at four doses (1.0, 2.0, 3.0 and 4.0g), and were compared with dimethylphthalate, a standard synthetic repellent, at the same doses under laboratory conditions (28-32^{0}C and 65-75 % R.H). The apparently un-infested cowpea seeds used as substrate were kept in a refrigerator under freezing conditions for four days; this is to kill any residual weevil in the seeds in order to safeguard against unwanted weevil infestation. The seeds were then equilibrated to the ambient laboratory conditions for five days prior to the test.

The disinfested cowpea seeds were weighed in batches of 200g and mixed thoroughly with 1.0-4.0g of the powder of each plant material (1.0, 2.0, 3.0 and 4.0g per 200g wt: wt, powder: substrate). Dimethylphthalate (1.0-4.0g) was also mixed thoroughly with 200g cowpea substrate. Three replicates were prepared for each treatment and the control. The admixtures were transferred to the bioassay cups designated for the doses.

Twenty unsexed adult F_1 *C. maculatus* were carefully picked from the culture with aspirator and were released through a long-stemmed funnel into each bioassay cup of substrate powder admixture. A control experiment consisting of 200g cowpea substrate and twenty adult *C. maculatus* without powder was set up. The cowpea weevils were exposed to treatments for 168 hours for each plant dose. Repellency was observed every twenty-four hours for 168 hours (a seven-day exposure period) according to FAO Bulletin (1999) for all plant types at different concentrations.

2.6. Repellency Test with the Plants' Extracts

Repellency of the experimental plants' extracts against *C. maculatus* at various concentrations (50.0, 75.0 and 100.0mg^{-ml}) was determined by filter paper repellency method (McDonald *et al.*, 1970). Filter papers (Whatman no. 1) were laminated with aluminium foil and were cut into equal halves and separated into two groups (A and B). Group A filter papers were further divided into three portions marked as A_1, A_2 and A_3. The latter were treated with 50.0, 75.0 and 100mg^{-ml} extracts, respectively. Each treated paper was made triplicate and air-dried overnight to evaporate the ethanol solvent. Group B half filter papers were not treated and served as control. The treated and un-

treated half filter papers were carefully attached, edge to edge, lengthwise with sellotape on the reverse side to produce full filter papers. Each full filter paper was placed in a petri dish with the seams of the papers oriented in one of three randomly selected different directions to avoid any incidental stimulus that could affect the distribution of the weevils.

Ten adult unsexed *C. maculatus* were released into the centre of each full filter paper in the petri dishes before covers were placed over them. Repellency was observed at every hour for five hours. Mean of percentage repellency was calculated, and the values were used to assign repellency class for the tested plant materials using Jilani and Su scale (1983).

2.7. Statistical Analysis

The percentage repellency was calculated at every twenty-four hours of exposure for each plant species using the equation:

$$\text{Repellency (\%)} = 100\left(\frac{\sum n_1 - n_2}{n_3}\right)$$

$\sum$ = Summation; n_1 = Initial number of weevils per replicate; n_2 = Final number of weevils per replicate; n_3 = total number of weevils per triplicate treatment. The data obtained were subjected to Analysis of Variance (ANOVA) for treatment means comparison; significant differences for treatment means were compared at 0.05 significant level.

3. Results

3.1. Repellent Effects of Plants' Powder Formulation

The strongest repellent effect against *C. maculatus* across doses (1.0 - 4.0 g) over 168 hours of exposure was *C. millenii*, followed by *Z. officinale*, *A. sativum*, *X. aethiopica* and *M. myristica*; the range of the cumulative percentage repellency at the lowest (1.0 g) and highest dose (4.0 g) were 75.0 - 98.3, 70.0 - 96.6, 73.3 - 95.0, 61.6 - 90.0 and 60.0 - 88.3, respectively. All the tested plants' powders, except *M. myristica* at low doses, were comparable and/or more repellent against *C. maculatus* than dimethyl phthalate, the standard repellent (Table 1). The ranking for these plant powders is: *C. millenii* (98.3 %) > *Z. Officinale* (96.6 %) > *A. sativum* (95.0 %)> *X. aethiopica* (90.0 %) > Dimethylphthalate (88.3 %) = *M. myristica* (88.3 %). The cumulative mean repellency of *C. maculatus* was affected by the dose of the plants' powders over 168 hours (Figure 1). Statistically, a two-way Analysis of variance (ANOVA) showed a significant difference ($P<0.05$) in the concentrations ($F = 649.50$; $P = 7.4x10^{-21}$) of the plants' powders and plants' species ($F = 5.33$; $P = 0.002$) used, suggesting that plant species' powders and concentrations had significant effects on *C. maculatus* repellency.

Results of the probit analysis for median repellencies (RC_{50}) of *C. maculatus* allowed determining the minimum concentration required to repel 50 % of the weevils as 0.91, 1.05, 1.15, 1.28, 1.09 and 1.39 g concentrations of *C. millenii, Z. officinale, A. sativum, X. aethiopica,* dimethylphthalate and *M. myristica*, respectively; in terms of the weevils mortality (Figure 1). Furthermore, the regression equations of the five plants suggested that the regression repellency (correlation coefficient) of *C. maculatus* and concentration of plants' powders is highly significant ($P<0.001$).

On the other hand, the effect of the different plants' dusts on the pests varied resulting in significant differences ($p<0.05$) in the mean repellency of the pest weevil over the exposure period of 168 hours (Table 2). The repellency ranking of these plants' powders against *C. maculatus*, showed the efficacy of these powders as repellent agents in the following order: *C. millenii* (89.6 %) > *Z. Officinale* (86.2 %) > *A. sativum* (82.5 %) > *X. aethiopica* (82.1 %) > *M. myristica* (76.6 %).

Table 1. Overview of cumulative percentage repellency (Mean ± S.E) of *Callosobruchus maculatus* exposed to 1.0-4.0g plant powders over 168 hours*****

Dose (g)	Plants' Powders/*C. maculatus* repellency					
	C. millenii	*Z. officinale*	*A. sativum*	*X. aethiopica*	Dimethylphthalate	*M. myristica*
0.0	0.04 ± 0.002 (4.0)	0.04 ± 0.002 (4.0)	0.04 ± 0.002 (4.0)	0.04 ± 0.002 (4.0)	0.04 ± 0.002 (4.0)	0.04 ± 0.002 (4.0)
1.0	0.75 ± 0.1 (75.0)	0.7± 0.09 (70.0)	0.73 ± 0.09 (73.3)	0.61 ± 0.07 (61.6)	0.73 ± 0.09 (73.3)	0.6 ± 0.07 (60.0)
2.0	0.91 ± 0.11 (91.6)	0.86 ± 0.11 (86.6)	0.78 ± 0.1 (78.3)	0.83 ± 0.1 (83.3)	0.8 ± 0.1 (80.0)	0.76 ± 0.09 (76.6)
3.0	0.93 ± 0.12 (93.3)	0.91 ± 0.11 (91.6)	0.83 ± 0.11 (83.3)	0.86 ± 0.11 (86.6)	0.86 ± 0.11 (86.6)	0.81 ± 0.1 (81.6)
4.0	0.98 ± 0.12 (98.3)	0.96 ± 0.11 (96.6)	0.95 ± 0.12 (95.0)	0.9 ± 0.11 (90.0)	0.88 ± 0.11 (88.3)	0.88 ± 0.11 (88.3)

*****Each percentage (in parenthesis) is mean of triplicate observations with 20 weevils per replicate

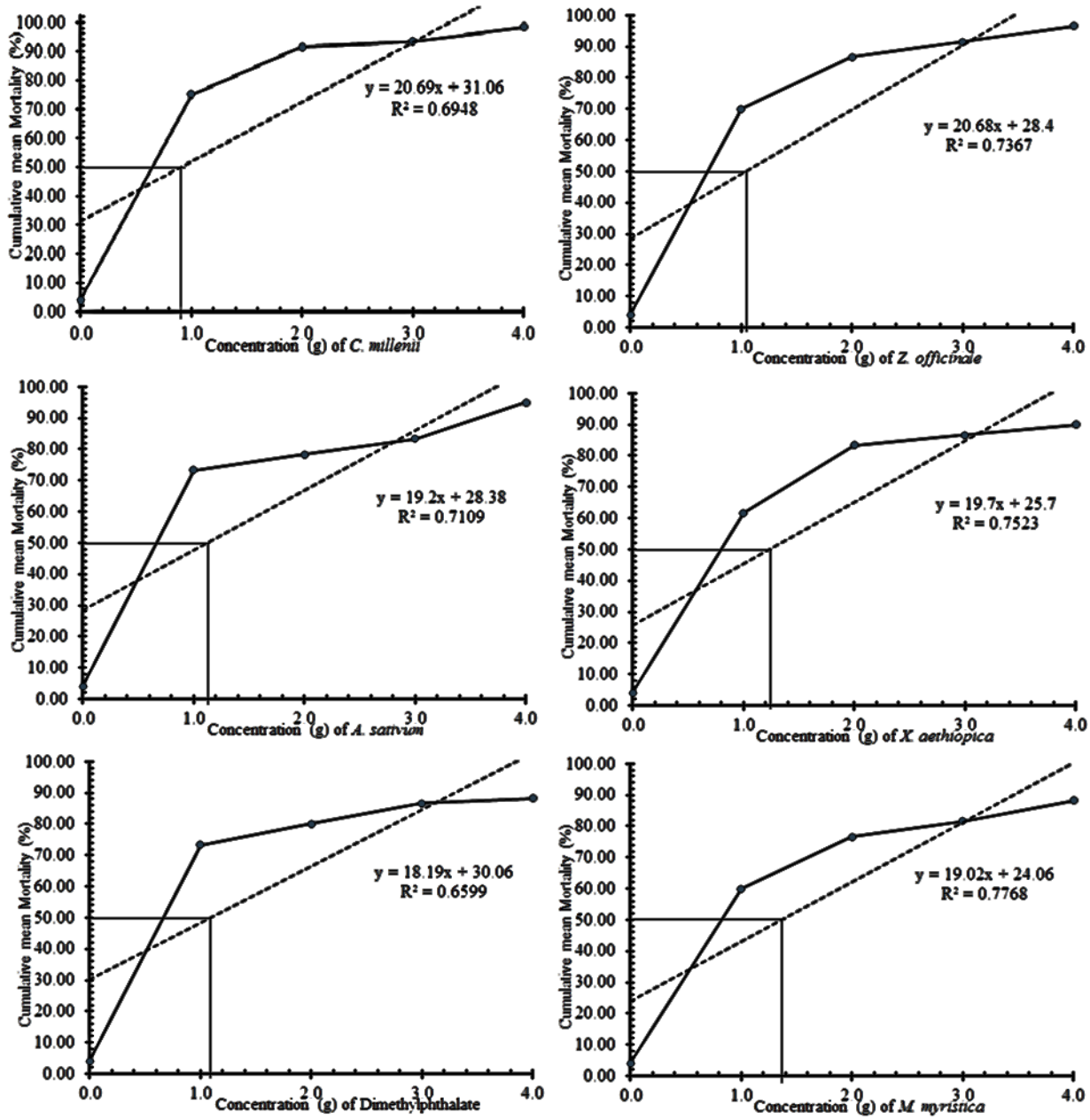

Figure 1. Median cumulative percentage repellency (RC_{50}) of *Callosobruchus maculatus* exposed to five (5) plants' powders at 168 hours post-treatment with regression equations

Table 2. Ranking of repellent potentials of experimental plant powders on *Callosobruchus maculatus* after 168 hours exposure

Plant Powder	Repellency Ranking (%)				
	1st	2nd	3rd	4th	5th
C. millenii	89.6				
Z. officinale		86.2			
A. Sativum			82.5		
X. aethiopica				82.1	
Dimethylphalate				82.1	
M. myristica					76.6

3.2. Repellency of Plants' Extracts

The repellency of *C. maculatus* to the plants' extracts of different concentrations over five hours is indicated in the following trend (Table 3): At 50.mg^{-ml}: *C. millenii* (76.6 %) > *X. aethiopica* (63.3 %) > *Z. officinale* (60.0 %) > *A. sativum* (53.3 %) > *M. myristica* (46.6 %) > Dimethylphthalate (43.3 %) > Control (3.3 %). At 75mg^{-ml}: *C. millenii* (86.6 %) > *X. aethiopica* (80.0 %) > *Z. officinale* (76.6 %) > *A. sativum* (66.6 %) = *M. myristica* > Dimethylphthalate (46.6 %) > Control (3.3 %). At 100.mg^{-ml}: *C. millenii* (90.0 %) > *X. aethiopica* (83.3 %) = *Z. officinale* > *M. myristica* (73.3 %) > *A. sativum* (66.6 %) = Dimethylphthalate > Control (3.3 %). The cumulative mean percentage repellency produced at the lowest (50.0mg^{-ml}) and highest (100. 0mg^{-ml}) extract concentrations against *C. maculatus* five hours after treatment was in the range of 76.6 - 90.0, 63.3 - 83.3, 60.0 - 83.3, 46.6 - 73.3 and 53.3 - 66.6 % for the extracts of *C. millenii*, *X. aethiopica*, *Z. officinale*, *M. myristica* and *A. sativum*, respectively (Table 3). Furthermore, data

analysis showed a significant difference ($p<0.05$) in mortality due to the concentrations of plants' extracts ($F= 145.36$; $df = 3$; $P= 2.62x10^{-11}$) and plants' species ($F = 6.51$; $df = 4$; $P = 0.0021$), suggesting that the concentration and plant species had a significant repellent effect on *C. maculatus*.

Table 3. Survey of cumulative mean percentage repellency of *Callosobruchus maculatus* exposed to different concentrations of plants' extracts 5 hours post-treatment

Conc. (mg^{-ml})	Experimental Plants' Extracts/ Repellency (%)					
	C. millenii	*X. aethiopica*	*Z. officinale*	*M. myristica*	*A. sativum*	Dimethylphthalate
0.0	3.3	3.3	3.3	3.3	3.3	3.3
50.0	76.6	63.3	60.0	46.6	53.3	43.3
75.0	86.6	80.0	76.6	66.6	66.6	46.6
100.0	90.0	83.3	83.3	73.3	66.6	66.6

The mean percentage of the repellency of the plant extracts was in the following decreasing order: *C. millenii* > *Z. officinale* > *X. aethiopica* > *A. sativum* > *M. myristica* versus the Dimethylphthalate and the control of 45.4 % and 3.3 %, respectively. Similarly, the repellency ranking of the plants' extracts (100.0 mg^{-ml}) was in the same order: *C. millenii* > *X. aethiopica* = *Z. officinale* > *M. myristica* > *A. sativum* (Table 4). However, all the extracts were more repellent than dimethylphthalate.

Table 4. Repellency status of plants' extracts in 100 mg^{-ml} treatment of *Callosobruchus maculatus* over 5 hours

Plants' Extracts	Repellency Status (%)				
	1st	2nd	3rd	4th	5th
C. millenii	90.0				
X. aethiopica		83.3			
Z. officinale		83.3			
M. myristica			73.3		
A. Sativum				66.6	

Data analysis showed that 50 % of *C. maculatus* were repelled at the application of 41.30, 47.50, 49.95, 60.05, 60.0 and 74.10 mg^{-ml} concentration of *C. millenii, X. aethiopica, Z. officinale, M. myristica, A. sativum* and dimethylphthalate, respectively (Table 5). Similar to the powder treatments, the correlation coefficients of *C. maculatus* and plants' extracts were highly and positively significant ($P<0.001$).

Table 5. Regression equation, correlation coefficient and median repellency (RC_{50}) of *Callosobruchus maculatus* over 5 hours of plants' extracts treatment

Plants' Extracts	Regression equation	Spearman correlation (r^2)	Correlation (%)	RC_{50}
C. millenii	y = 0.8954x + 13.757	0.8717	87.17	41.30
X. aethiopica	y = 0.8344x + 10.54	0.919	91.90	47.50
Z. officinale	y = 0.8265x + 9.3086	0.9427	94.27	49.95
M. myristica	y = 0.7275x + 6.5257	0.9698	96.98	60.05
A. Sativum	y = 0.6663x + 9.9714	0.8936	89.36	60.40
Dimethylphthalate	y = 0.6091x + 5.6857	0.9622	96.22	74.10

4. Discussion

Plant species having a repellent property prevent pest damage of food like cereals and legumes as well as other valuable substances in storage by rendering them unpalatable or offensive to the pests, thereby, making the pests avoid such materials. The repellent effectiveness of the powder and crude extracts obtained from the tested plants: *A. sativum, C. millenii, M. myristica, X. aethiopica,* and *Z. officinale* against *C. maculatus*, was determined under laboratory conditions using the cup bioassay and filter paper repellency methods. The results of the study indicated that all the plants' dust constituted effective repellents of the insect pest at the doses (1.0 – 4.0 g) and concentrations (50.0 – 100.0mg^{-ml}). Except for *M. myristica*, all the other plants' dust produced a higher percentage repellency values than dimethylphthalate, the standard repellent used in this study. However, the effect of the different plants' dust on the pests varied resulting in significant differences ($p<0.05$) in the mean repellency of the pests over the exposure period of 168 hours. Repellency of the pest increased gradually throughout the test period. The results indicated that the highest repellency of the pest across the plants' types over 168 hours was produced by the highest treatment of 4.0g/200g (Table 1). These findings agreed with the study of Egwunyenga *et al*., (1997) who reported that the powders and crude extracts of alligator pepper (*Aframemum melagueta*) were most repellent against *C. maculatus* at the highest dose of 0.6/20g substrate; this dose approximated to 0.6g/30g substrate in the current study.

The results of this study indicated that the *M. myristica* powder produced the lowest percentage repellency values against *C. maculatus*. *M. myristica* is strongly aromatic in taste (Oparaeke and Dike, 2005) and has a delightful fragrance due to its essential oil consisting mainly of p-cymene (31.5 %), α-phellandrene (18.1 %), α-pinene (6.1 %), and β-piene (5.1 %) (Owolabi *et al*., 2009). These are terpene hydrocarbons, terpene derivatives, and phenylpropanoid. Despite the possession of these chemical constituents, the *M, myristica* powder was the least repellent. One plausible reason for this phenomenon may be the ease with which nutmeg loses its fragrance when pulverized into dust. It is, therefore, important that the necessary amount of nutmeg powder to be used should be grated from a whole nutmeg and used immediately to prevent loss of potency. Alternatively, it may be expedient to apply nutmeg as a whole undamaged nut instead of grinding, and to use it as powder in order to achieve its full potential as a repellent.

The extracts and powders of *X. aethiopica* in this study ranked second and fourth with respective repellency values of 83.3% and 82.1%. The high repellency values recorded might be attributed to the presence of toxic complex compounds like terpenes and their derivatives (Pérez *et al*., 2010) among which are terpinen-4-ol, β-pinene, ά-terpineol, sabinene, 1,8-cineole, myrtenol and kaurane derivatives (Ito *et al*., 2018). The toxicity of terpenes in *X. aethiopica* against the *Sitophilus oryzae* (Byung-Ho *et al*., 2001) and *C. maculatus* (Ito *et al*., 2018) have been documented. Similarly, Keane and Ryan (1999) have established that terpenes' derivatives affect the nervous system of the wax moth (*Galleria melonella*) by inhibiting the enzymatic activities of acetylcholinesterase.

C. millenii was the most superior repellent of *C. maculatus*. This may be attributed to its pungent aroma due to the active chemicals (Sabinene, pinene, camphene among others) in the plant material. The repellent activity of garlic (*A. sativum*) against *C. maculatus* may be ascribed to its various sulphur compounds which are

responsible both for garlic pungent odour and many of its pharmacological properties. Garlic contains sulphur compounds and other chemical constituents which may be responsible for the repellency in this study. Negro pepper contains essential oils which consist of beta-pinene, 1,8-cineol and alpha-terpineol (Motheshard, 2009).

The powders and extracts of *Z. officinale* rhizome ranked second after *C. millenii* in terms of repellency against *C. maculatus* at the different concentrations used. The major phytochemicals in *Z. officinale* are α-zingiberene (28.9 %), β-sesquiphellandrene (13.1 %), z-γ-bisabolene (12.5 %) and arcurcumene (11.3 %) (Owolabi *et al*., 2009). The monoterpenoids (R)-linalool and (S)-2-heptanol found in the *Z. officinale* oil extracts and other monoterpenoids and Citral have been shown to be good repellents to *Tribolium castneum* and *Rhyzopertha dominica* (Ukeh and Umoetok, 2011). These chemicals in the test plants may be responsible for the pungent odour that repelled the insect pest. The repellency results of 86.2 % (powder) and 83.3 % (extract) offered by *Z. officinale* in this study are comparable to Ogbonna *et al.* (2016) who found the *Z. officinale* powder and oil to cause a repellency of 100 % to *C. maculatus* after four days at the application of 700 µl/mL. The repellency of insect pests may generally be attributed to the chemosensory effects of plants' secondary metabolites as terpenes which insects take up through their respiratory system (Xie *et al*., 1995).

In this study, *C. maculatus* responded differently to the repellent effects of the plants' products in accordance with its behavioural tendency. The weevil is a fast moving insect (Mohan and Field, 2002). Hassanli *et al*., (1990) reported the effective repulsion of *C. maculatus* to wild basil plant (*Ocimum suave*) one hour after treatment. The high percentage emigration may be attributed to the fast movement of the pest from the substrates.

This current study indicated further that repellency was more dependent on concentration than exposure time. Dose-dependent repellency has been reported by Mohan and Field (2002) and Egwunyenga *et al*., (2000). Furthermore, the repellency of the pests was relatively higher during the first forty-eight hours of exposure, and thereafter, an additional repellency value decreased progressively. This may probably be attributed to the loss of potency due to the vapourization of the active chemicals in plants' products.

5. Conclusion

The use of botanical products prevents several insect pests from infesting stored food products. The five plants tested in this study exhibited varying degrees of repellency against *C. maculatus*. On this basis, local farmers are advised to use these plants' materials to protect cowpea seeds in storage against weevil infestation. Based on the efficacy of the plants used, it is recommended that similar investigations of different plants' species be carried out to further ascertain their efficacy against *C. maculatus* or/and other storage pests. It is, therefore, expedient to control weevil populations to a tolerable limit in storage since a higher bruchid population results in a higher level of stored grain damage.

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Conflicts of interest:

There are no conflicts of interest.

References

Al-Moajel NH and Al-Fuhaid WL. 2003. Efficacy and persistence of certain plant powders against Khapra beetle, *Trogoderma granarium*. Everts, Fayoum *J Agric Res Dev.,* **17**: 107-114.

Boeke SJ, Baumgart IR, van Loon JJA, van Huis A. 2004. Toxicity and repellence of African plants traditionally used for the protection of stored cowpea against *Callosobruchus maculatus. J Stored Prod Res.*, **40(4)**:423-438.

Byung-Ho L, Won-sik C, Sung-Eun L, and Byeoung-Soo P. 2001. Fumigant toxicity of essential oils and their constituent compounds towards the rice weevil, S*itophilus oryzae* (L). *J Crop Protect.,* **20**: 317-320

Caswell GH. 1981. Damage to stored cowpea in the Northern part of Nigeria *Samaru J Agric Res.*, ***1***, 11-19.

Cherry JE, Bantino A, Djegul D and Lomers C. 2005. Suppression of the stem borer *Sesamia catamistis* (Lepidoptera: Noctuidae) in maize following seed dressing, topical application and stem injection with African isolates of *Beauveria bassiana. Int J Pest Mgt.*, **50**: 67-73.

Egwunyenga AO, Nmorsi OPG and Dibie C. 1997. Repellent effects of *Aframemum melagueta* (K) (Anonaceae) to *C. maculatus* (F) (Coleoptera: Bruchidae) *Bull Sci Assoc Nig.,* **21**: 163-166.

Egwunyenga OA, Nmorsi OPG and Alo EB. 2000. Repellency of two pepper varieties to *cowpea weevil* (*Callosobruchus maculatus*). *Nig J Sci & Environ.,* **2**: 69:73.

Ewete F, Nicol RW, Hengsanwad V, Sukumalanand P, Satasook C, Isman MB, Arnason JT. 1996. Insecticidal activity of *Aglaia odorata* extract and the active principle rocaglamide to the corn borer, *Ostrinia nubilalis* Hubn (Lep., Pyralidae). *J Applied Entomol.,* **120**: 483-488.

FAO. 1999. The use of spices and medicinal as bioactive protestant for grains. www.fao.org/docrep/12230c/x2230c05.htm accessed 15-06-18.

Gbaye OA, Millard JC, Holloway GJ. 2011. Legume type and temperature effects on the toxicity of insecticide to the genus *Callosobruchus* (Coleoptera: Bruchidae). *J Stored Prod Res.,* **47**: 8–12.

Hassanli LW, Ole-Sitayo N, Moreka L, Nokoe S and Chapyg S. 1990. Weevil repellent constituents of *Ocimum suave* leaves and *Caryophyllata* cloves used as grain protectants in parts of East Africa. *Discov & Innov.,* **2(2)**: 91-95.

Ito EE and Ighere EJ. 2017a. Bio-insecticidal potency of five plant extracts against cowpea weevil, *Callosobruchus maculatus* (F.), on stored cowpea, *Vigna unguiculata* (L). *Jordan J Biol Sci.,* **10(4)**: 317-322.

Ito EE and Ighere EJ. 2017b. **Basic Entomology and Pest Control**. 1st Ed., University Printing Press, Delta State University Abraka, Nigeria. ISBN: 978-33772-08-12, 361 pp.

Ito EE and Ukpohwo AR. 2018. Termiticidal Efficacy of *Citrus* Peel Extracts against Termites (*Macrotermes bellicosus*). *J Biol Studies,* **1(3)**: 98-105.

Ito EE, Ukpohwo AR and Okiriguo VI. 2018. Insecticidal activity of *Xylopia aethiopica* (Family; Annonaceae) against *Callosobruchus maculatus* (F) (Coleoptera: Bruchidae) and *Sitophilus oryzae* (Coleoptera: Curculionidae). *J Biol Studies,* **1(3)**: 106 – 115.

Ito EE and Utebor EK. 2018. Insecticidal Toxicity of Goat Weed, *Ageratum conyzoides,* Linn. (Asteraceae) on Smoked Fish Weevil, *Dermestes maculatus,* Degeer (Coleoptera: Dermestidae). *Jordan J Biol Sci*., **11(2)**: 223 – 229.

Jilani G and Su HCF. 1983. Laboratory studies on several plant materials on insect repellants for protection of cereal grains. *J Entomol Soc Am.,* **76(1)**: 154-157.

Keane S and Ryan MF. 1999. Purification, characterization, and inhibition by monoterpenes of acetylcholinesterase from the wax moth, *Galleria mellonella* (L.). Insect Biochem & Mol Biol., **29**:1097–1104.

Kumar PP, Mohan S, and Balasubramanian G. 2004. Effects of whole pea flour and a protein-rich fraction as repellants against stored-product insects. *J Stored Prod Res.,* **40**: 547-552.

McDonald LL, Guy RH and Speirs RD. 1970. Preliminary evaluation of new candidiate materials as toxic, repellants and attractants against stored product insects. *USAD* Mark Rep., 882, 8.

Mohan S and Fields PG. 2002. A simple technique to assess compounds that are repellent or attractive to stored- product insects. *J Stored Prod Res.,* **38**:*23-31*.

Motheshard TD. 2009. http/www. Herballegacy.com/ motheshard _chemical.html.

Ofuya TI. 1991. Observations on Insect Infestation and Damage in Cowpea (*Vigna Unguiculata*) Intercropped With Tomato (*Lycopersicon Esculentum*) in a Rain Forest Area of Nigeria. *Exp Agric.*, **27**: 407-412.

Ogbonna CU, Okonkwo NJ, Nwankwo EN, Okeke PC, Ebi SE. 2016. Bioefficacy of *Zingiber officinale* against *Callosobruchus maculatus* Fabricius (Coleoptera: Bruchidae) infesting cowpea. *Intl J Entomol Res.,* **1(4)**: 19-25.

.

Okonkwo EU and Okoye WL. 1996. The efficacy of four seed powders and essential oils as protectants of cowpea and maize grains against infestation by *Callosobruchus maculatus* (Fabricius) (Coleoptera: Bruchidae) and *Sitophilus zeamais*(Motschulsky) (Coleoptera: Curculionidae) in Nigeria. *Int J Pest Mgt.,* **42***: 143-146.*

Oparaeke AM and Dike MC. 2005. *Monodora myristica* (Gaertn), (Myristicaceae) and *Allium cepa* (Lilliaceae) as protectants against stored cowpea seed Bruchid *(Callosobruchus maculatus)* Infestation. *Nig J Entomol.,* **22**: 84-92.

Owolabi MS, Oladimeji MO, Lajide L, Singh G, Marimuthu P and Isidorov V. 2009. Bioactivity of Three Plant Derived Essential Oils against The Maize Weevils *Sitophilus zeamais* (Motschulsky) and cowpea weevils *Callosobruchus maculatus* (Fabricius). *EJEAFChe,* **8 (9)**: 828-835.

Pérez SG, Ramos-López MA, Zavala-Sánchez MA and Cárdenas-Ortega NC. 2010. Activity of essential oils as a biorational alternative to control coleopteran insects in stored grains. *J Medicinal Plants Res.,* **4(25)**: 2827-2835.

Srivastava KM and Pant JC. 1998. Growth and developmental response of *C. maculatus* (Fabr.) to different pulses. *Indian J Entomol.,* **51**:269-272.

Tanzubil PB. 1991. Control of some insect pests of cowpea *(Vigna unguiculata)* with neem *(Azadirachta indica)* in Northern Ghana. *Trop Pest Mgt.,* **37***:* 216-217

Ukeh DA and Umoetok BA. 2011. Repellent effects of five monoterpenoid odours against *Tribolium casteneum* and *Rhyzopertha dominica* in Calabar, Nigeria. *J Crop Prot.,* **30**:1351-1355.

Uyi OO and Obi BN. 2017. Evaluation of the Repellent and Insecticidal Activities of the Leaf, Stem and Root Powders of Siam weed (*Chromolaena odorata*) against the Cowpea Beetle, *Callosobruchus maculatus. J Appl. Sci. Environ. Mgt.,* **21(3)**: 511-518.

Xie YS, Fields PG, and Isman MB. 1995. Repellency and toxicity of azadirachtin and neem concentrations to three stored product beetles. *J Econ Entomol.,* **88***:* 1024-1031.

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Advanced and Rapid Serodiagnosis of Oestrosis (*Oestrus ovis*; Diptera: Oestridae) in Sheep Using Indirect and Dot-ELISA

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Abstract

This study is aimed at evaluating performance of the indirect ELISA for the diagnosis of oestrosis versus the rapid easy assay of Dot- ELISA which could be directly used in the field. Two hundred and forty head of sheep were examined in a Cairo abattoir over the period from May 2017 to May 2018. Sera were collected from each examined sheep and preserved in -20 °C. The Anterior cone (AC) of third-stage larvae of *Oestrus ovis* were prepared. Hyperimmune sera were processed in rats using the anterior part of the larvae (AC); ELISA and Dot- ELISA tests were done after checkerboard titration. The lowest antigen concentration which gives positive results in ELISA was 10 µg protein, whereas the concentration used in the Dot- ELISA was 200 ng/µL, with the sera dilution being 1:100 in the two tests. The two tests were performed with known *O. ovis*-positive and negative sheep sera and known hyperimmunized rat sera against AC of *O. ovis*, as well as known sera for *Coenurus cerebralis*, *Dictyocaulus filaria* and *Haemonchus contortus* without an *O. ovis* infection. Two hundred out of the two-hundred and forty examined sheep were positive for *O. ovis* larvae at post mortem. High optical density (O.D.) values ranging from 0.9- 2 were estimated in the sheep infected with the second and third-stage larvae. On the other hand, O.D. values varying from 0.9 to 1.6 were recorded when sheep (95 sheep) were infected only with the third-stage larvae of *O. ovis*. The results of the current study confirm that Dot-ELISA had similar sensitivity and specificity to those by the indirect ELISA but is more rapid and has an easy assay. So, it could be applicable in the field directly for diagnosing oestrosis.

Keywords: *Oestrus ovis*, Nasal bot fly, ELISA, Dot-ELISA, Coenurosis, and Lungworm.

1. Introduction

Oestrosis has serious pathogenic effects on small ruminants (goats and sheep) as the adult flies deposit its first larval stage in and around the nose of ovine and caprine species. The first-stage Larvae wander in the nasal passages, sinuses, and ethmoid bones in which they molt to the second and third larval stages. Then, the full mature third-stage larvae excrete to the outside by sneezing onto the ground where they become pupa in the soil and complete their life cycle (Hall and Wall, 1995, Zumpt, 1965). The migration and development of the larvae inside their habitat (nasal cavities and sinuses) cause serious problems to small ruminant animals which may lead to several pathological conditions such as nasal discharges, frequent sneezing, and respiratory disorders resulting in serious economic losses (Pandey and Ouhelli, 1984; Dorchies *et al*., 2000).

Different epidemiological studies on *O. ovis* larvae recorded their wide distribution all over the world. These parasites were reported present in different countries by (Jagannath *et al*., 1989, Alahmed, 2000, Abo-Shehada *et al*., 2000; Karatepe *et al*.2014; Ozdal *et al*.2016; Allaie *et al*.2016) in India, Saudia Arabia, Northern Jordan, Turkey, Kashmir respectively. Different records also confirmed their distribution in African countries (Biu and Nwosu, 1999, Amin *et al*., 1997, Berrag *et al*., 1996) such as Nigeria, Egypt and Morocco respectively. There are also records of their distribution in some European countries including Greece (Papadopoulos *et al*., 2001), France (Yilma and Dorchies, 1991; Bergeaud *et al*., 1994; Dorchies *et al*., 2000), Italy (Caracappa *et al*., 2000; Scala *et al*., 2001), South-western Germany (Bauer *et al*., 2002), Spain (Reina *et al*., 2001; Alcaide *et al*. 2003). In other parts of the world, the infection was recorded in Mexico (Martinez *et al*., 1999), in Argentina by (Trezeguet, 1996), and in the United Kingdom by (Bates, 1997; Goddard *et al*., 1999).

Many cases of Ophthalmomyiasis in humans were recorded through the infection of the eyes by the first-stage larvae of *O. ovis* (Lucientes *et al*., 1997; Pampiglione *et al*., 1997; Prosl and Meyer, 2003; Fasih *et al*. 2014). Masoodi and Hosseini (2003) reported another case of pharyngeal oestrosis by the first-stage larvae.

Oestrosis is endemic in Egypt due to the hot and dry weather which constitutes good conditions for the fly and its larvae to grow. Unfortunately, the detection of *O. ovis* antibody levels is still poorly investigated in Egypt. So, the target of this study is to assess and compare the performance of ELISA in the diagnosis of *O. ovis* infection in sheep sera with the performance of the new and rapid

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assay of Dot-ELISA used in the diagnosis of this parasitism directly in the field.

2. Materials and Methods

2.1. Collection of Samples

Two hundred and forty head of sheep were examined in a Cairo abattoir over the period from May 2017 to May 2018 (twenty sheep per month). The Sagittal section of sheep heads was performed, and the larvae were collected from the nasal passages and at the base of the horns (Figure 1). All the collected larvae were identified according to Zumpt, (1965). A total of fifty larvae from sheep were used in the serological analysis. Blood was collected from each examined sheep and centrifuged. The sera were collected in sterile vials and stored at -20 °C until used. In addition to *O. ovis* sera, three parasites were chosen; two being the main causes of respiratory diseases and one causing a nervous manifestation in sheep. The sera of these sheep positively-infected with those parasites were chosen during postmortem in the abattoir; the sheep infected with only one parasite only, either with *Coenurus cerebralis* (*C. cerebralis*), *Dictyocaulus filaria* (*D. filaria*) and *Haemonchus contortus* (*H. contortus*) without *O. ovis* larvae infection. Negative control serum was obtained from one- month old sheep.

2.2. Antigen Preparation

The Anterior Cone (AC) of third-stage larvae (L3) were dissected freshly, relaxed in an iced PBS medium under a stereoscope microscope (LEICA M60, USA) for the cutting of the anterior part of L3 just after the cephalopharyngeal skeleton which had the larval cuticle and salivary gland. The antigen (AC) was preserved in 1 ml PBS (PH 7.2). The AC of L3 was ground using a homogenizer, and sonication was then done for five minutes at a 10-pulse rate 60-80 amplitude value using cole parner ultrasonic sonicator. The homogenates were centrifuged at 14,000 RPM for twenty minutes at 4 °C as described by Innocenti *et al*. (1995) and Angulo- Valadez *et al*. (2008). The supernatant was collected and preserved for further analysis. The protein content of the prepared antigen (AC ag) was determined by the method of Lowry *et al*., (1951). Then, the antigen (AC) was stored at -20 °C until used in the two diagnostic tests (ELISA; Dot-ELISA).

2.3. Preparation of Hyperimmune Sera

Four male rats (*Rattus norvegicus albino*) of about a 150 gram in weight were raised for the preparation of hyperimmune sera versus AC according to Innocenti *et al*. (1995) with some modification. The rats were housed in two groups (two control negative and two immunized against AC ag). The housing was in a conventional rat cage with straw bedding. The rats were supplied with normally balanced rats' pellet and supplied with water *ad libitum*. The rats were raised in ambient temperature and humidity. The immunized rats were injected with 1mg of protein AC, mixed in 1ml of mineral oil subcutaneously (1^{st} dose). Then, two subsequent intramuscular injections of 0.5 mg of protein antigens in the same volume of mineral oil were injected at a two-week interval. The rats were slaughtered for the collection of sera two- weeks after the last dose. The collected hyperimmune rat sera were stored at the refrigerator until used. Ethical approval was obtained from the Animal Ethical committee of the Faculty of Veterinary Medicine, Cairo University under number: CU/II/F/105/18.

2.4. Sera Examined

The sera samples used in the ELISA and DOT- ELISA tests were the hyper-immunized rat sera against AC ag, negative control sera from rats and one-month old sheep, and sera from naturally infected sheep with *O. ovis*. As well as the three sera from sheep infected with other parasites such as *C. cerebralis* and *D. filaria*, *H. contortus* without *O. ovis* infection.

2.5. Indirect Enzyme-Linked Immunosorbent Assay (ELISA)

In order to find out the optimum assay dilution of the antigen and tested sera which could be used under known dilution of conjugate and substrate materials, checkerboard titration was done according to Harlow and Lane (1988). A ninety- six well, flat- bottomed ELISA plates were incubated overnight with 100 µL/ well of antigen at 10 µg /mL coating buffer (0.1 M Sodium carbonate, pH 9.6) in 4 °C. Two hundred µL/ well of the blocking buffer (Bovine serum albumin (BSA) - PBS) were added for two hours at 37 °C. After three times washings with PBS- Tween-20; 100 µL of diluted sera 1:100 in PBS were incubated in two wells (each serum sample was put in two wells to take the average) and were incubated at 37 °C for two hours. One hundred µL/ well of horseradish peroxidase anti-sheep IgG; anti-rat IgG conjugate (Sigma, A-5420) diluted at 1:1000 were added to each well and incubated for one hour at room temperature. A hundred microliters of a substrate buffer containing (10 mg Ortho-Phenylene Diamine (OPD)) diluted in a citrate buffer (pH 5.0) and 30 % H_2O_2 were added to the plate well. Finally, the ELISA plate was stopped by adding 100 µL of 3N H_2SO_4 (stopping buffer), and the absorbance values or Optical density (O.D.) were obtained from an ELISA reader (Bio-Rad, USA) at 450. The sera were positive when the absorbance values were as or more than the cut off value equal to a double fold of the mean negative sera, The ELISA was done according to Tabouret *et al*. (2001); Angulo- Valadez *et al*. (2009; 2011) and Attia *et al*. 2019.

2.6. Determination of ELISA Parameters

Different ELISA parameters were calculated according to Tabouret *et al*. (2001); Angulo- Valadez *et al*. (2009; 2011) and Attia *et al*. 2019. The sensitivity and specificity, positive predictive value, and the negative predictive value were recorded.

2.7. Dot - Enzyme-Linked Immunosorbent Assay (Dot - ELISA)

Checkerboard titration was done for the assessment of the optimal condition of the different solutions of ELISA (AC antigen; the sera and the conjugate). Two hundred ng of the AC antigen was dotted onto nitrocellulose membrane discs (NC) with 0.22 µm pores (Bio-Rad Laboratories, USA) placed in ELISA plate and incubated for one at 37 °C. The NC discs were blocked using blocking solution (BSA)-PBS for thirty minutes. After being washed with (PBS-T), for three times, positive and negative hyperimmune sera as well as the sera of sheep naturally infected with *O. ovis*, and three sera of three

other parasites mentioned in ELISA were diluted in PBS. One µL of 1:100 diluted sera was dotted onto NC. After one hour and three times of washing, the anti-rat IgG and anti-sheep IgG conjugated horseradish peroxidase (Sigma, A-5420) were diluted 1:1000 in PBS was dotted at 1µL/ well. After one hour, and another three times of washing to NC. The used substrate (4-chloro-1-naphthol and methanol in PBS containing 30 % H_2O_2) was added onto NC. The developed violet color was stopped by washing the NC with distilled water. The violet color produced indicates a positive result, while if no color was developed it is considered as a negative result. The Dot- ELISA was carried out according to Kumar *et al*. (2008); Lakshmanan *et al*. (2016); Paller *et al*. (2017).

2.8. Statistical Analysis

The linear regression was assessed to detect the possible evaluation of antibody titers in relation to the number of larvae for each positive animal. Pearson's correlation was performed to detect the variation in different parameters. The statistical analysis was performed by SPSS, version 11.5.1.

3. Results

All the collected larvae were identified as *O. ovis* second and third-stage larvae at the base of the horn, in the nasal passages and the sinuses. *Oestrus ovis* larvae were yellowish to brownish in color with dark transverse bands on the dorsal surface according to the stage. Second- stage larvae were yellowish in color, while third-stage larvae were dark yellowish to brownish in color with dark transverse bands on the dorsal surface, Figure1.

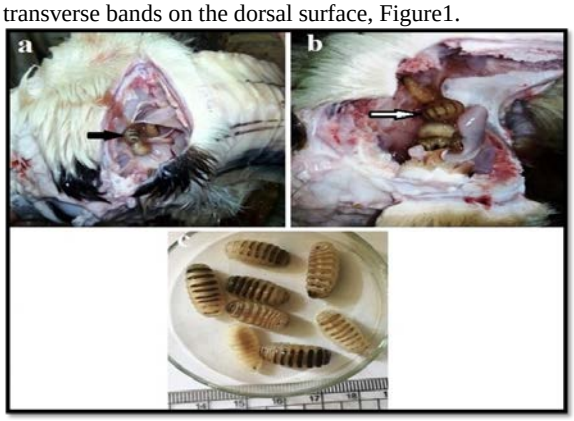


Figure 1. A, B, *O. ovis* larvae recovered from the sheep skull at the base of the horn and in the nasal sinuses. C. Collected *O. ovis* larvae note: it is oval in shape with a dark band on the dorsal surface.

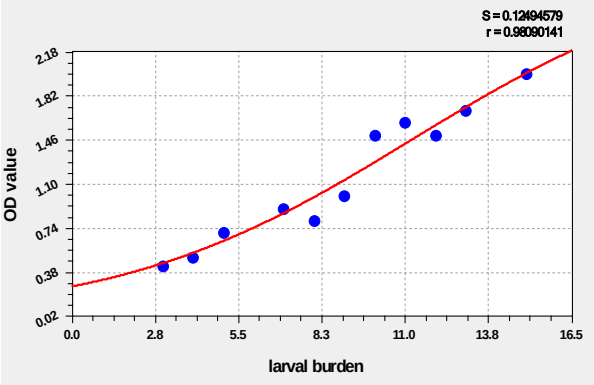
The lowest antigen concentration that gives positive results after checkerboard titration for indirect ELISA was 10 µg protein of AC antigen (ag), while it was 200 ng/µL in Dot ELISA, with the sera dilution being 1:100 in the two tests. The two tests were performed with known *O. ovis* positive and negative sheep sera and known hyper-immunized rat sera against AC ag of *O. ovis*, as well as known sera for *C. cerebralis*, *D. filaria*, and *H. contortus* without *O. ovis* infection.

Indirect-ELISA was used for the diagnosis of anti- *O. ovis* antibodies in the collected sera versus anterior cone (AC) antigen of third-stage larvae. Two hundred out of the two-hundred and forty examined sheep were positive for *O. ovis* at post- mortem. The sera of non- inoculated rats and one-month old sheep were used as a negative control while the hyperimmune rat serum inoculated with AC ag of third-stage larvae was used as a positive control.

The results displayed in Table 1 show that the optical densities (O.D.) varied according to the stage and number of larvae infecting sheep. High O.D. values ranging from 0.9- 2 were estimated in sheep infected with the second and third- stage larvae while O.D. varying from 0.9 to 1.6 were recorded when sheep (95 sheep) were infected only with the third-stage larvae of *O. ovis*. Sheep harboring only second stage larvae; O.D. value ranged between 0.5 and 0.7, Figure 2.

Table 1. ELISA Optical densities (O.D.) of sheep infected with *O. ovis* larvae in relation to larval stage and mean larval burden.

Stage of larvae	No. of infected sheep	Mean larval burden	Optical densities ± S. E
2nd only	25	5-20 (av.10)	0.5-0.7 (av. 0.59) ± 0.095
3rd only	95	10-30 (av. 20)	0.9-1.6 (av.1.078) ± 0.132
2nd and 3rd	80	20-30 (av. 25)	0.9- 2.0 (av. 1.55) ± 0.036
Total	200		



Logistic Model: y=a/(1+b*exp(-cx)) Standard Error: 0.1249458
Correlation Coefficient: 0.9809014

Figure 2. Effects of the mean larval burden of *Oestrus ovis* larvae on the mean optical densities obtained by indirect- ELISA

The results displayed in Figure 3 indicate that the optical densities (O.D.) are significantly correlated with the months of the year. From May to August, no significant correlation was estimated as the O.D. values ranged from (1.5 to 1.3); throughout this period, the third-stage larvae were collected. There was a significant decrease in O.D. values ($P < 0.05$) and mean larval burden during the months from August to September. No significant correlation was found between September and October. However, between October and November, there was a significant decrease and the O.D. value ranged from 0.9 to 0.5 ($P< 0.01$). While between November, December, and January, there was no significant correlation ($P> 0.05$) between these months. There was a significant increase ($P< 0.05$) in January and February, and no significant correlation was found between the other months.

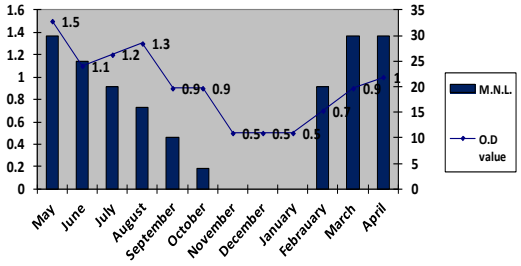


Figure 3. Effects of the mean number of *Oestrus ovis* larvae on the mean optical densities obtained by indirect ELISA during different months of the investigation period from May 2017 to May 2018; M.N.L.: Mean number of larvae.

Different parameters of indirect ELISA and Dot-ELISA are presented in Tables 2 and 3.

Table 2. Positive and negative values obtained from Indirect ELISA and Dot ELISA

Values \ Test	Indirect ELISA	Dot-ELISA
True Positive	198	199
False Negative	2	1
False positive	0	0
True Negative	40	40

Table 3. The diagnostic sensitivity, specificity, positive and negative predictive values as well as accuracy of the two tests for the diagnosis of oestrosis in sheep

Test / Statistics	Indirect-ELISA		DOT-ELISA	
	Value	95% CI	Value	95% CI
Sensitivity	99%	96.43%-99.88%	99.5%	97.25% - 99.99%
Specificity	100.00 %	91.19% - 100.00%	100.00 %	91.19%-100.00%
Positive likelihood Ratio	-	-	-	-
Negative likelihood Ratio	0.01	0.00- 0.05	0.01	0.00-0.04
Disease Prevalence	83.33%	78.00%-87.82%	83.33%	78.00%-87.82%
Positive Predictive Value	100.00%	-	100.00%	-
Negative Predictive Value	95.24 %	83.43% - 98.76%	97.56%	84.99%-99.65%
Accuracy	99.17%	97.02% - 99.90%	99.58%	97.70%-99.99%

Based on data of the present study, cross reactions were not detected in ELISA and Dot-ELISA by AC ag.

Sera from sheep infected with other parasites such as *C. cerebralis*, *D. filaria* and *H. contortus* without *O. ovis* infection; none of these sera were reacted with AC ag of *O. ovis* in indirect-ELISA and Dot-ELISA. Thus, no cross-reactions were recorded with this antigen (AC ag).

The results of Dot-ELISA could detect anti- *O. ovis* IgG antibodies at 200 ng/mL with 99.5 % sensitivity and 100 % specificity when postmortem examination of the skull and horn was fixed as the test standards.

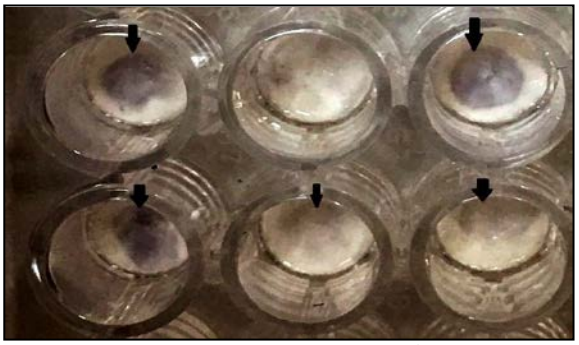


Figure 4. Dot-ELISA plate showing the wells of strongly positive sera represented by arrows, while the well with no arrows, shows the negative sera

The test standards revealed that two hundred sheep were positive for *O. ovis* larvae while in indirect ELISA and Dot-ELISA two and one sera were respectively false negative. No sera in the two tests give false positive results. The two tests confirm the test standards.

4. Discussion

Infection with *O. ovis* is very difficult to diagnose and could be misdiagnosed with other diseases which had a similar manifestation. Also, *O. ovis* larvae can't be diagnosed using blood smears or even fecal examination. So, the serological or molecular tests are the only tests which could diagnose oestrosis in sheep and goats.

Indirect ELISA was used for the diagnosis of anti- *O. ovis* antibodies in the collected sera versus anterior cone antigen of the third- stage larvae, in order to detect the systematic IgG which reacts with polypeptides of the larval cuticle of 56 KDa which prove to be the strongest antigenic materials. However, a salivary gland protein 28 KDa is also the most immunogenic fractions; this hypothesis was recorded by (Innocenti *et al*. 1995; Tabouret *et al*. 2001). So, this study used the anterior cone antigen of L3 which combines the salivary glands and cuticle antigen based on the results of the two previous authors.

In this study, the AC ag of L3 was used as an antigen for indirect ELISA and Dot- ELISA. The assay confirmed the results of the macroscopic survey (test standard) since positive results (O.D. varying from 0.5- 2) were recorded for all the infected sheep. Low optical density of 0.5- 0.7 was reported when L2 was only present, but when L2 and L3 infect sheep (O.D. 0.9- 2). According to the authors, the low O.D values associated with the second larval stage infection may be related to a short duration of the infection period of L2, the immunogenic nature of this larval stage, and/or the antigen used from L3; this gives good results in determining the stage present in the animals. Similar results were reported by Innocenti *et al*. (1995) who used the salivary glands' proteins of the *O. ovis* larvae to detect the antibodies' levels of these botfly larvae and stated that the salivary glands were the most immunogenic proteins in the infected sheep. However, this study confirms that the AC ag gives similarly high values to the results of the study using the salivary gland from L3 by Angulo-Valadez *et al*. (2009) in their study of the systemic IgG response using the salivary glands of L3.

Angulo- Valadez *et al*., (2011) studied the immunogenic reaction against oestrosis in sheep and detected a rise in Th2 immune response. Other studies on the immunogenic relationship between *O. ovis* and other concurrent helminths infection revealed that there is no cross-reaction between *O. ovis* larvae and other parasitic gastero-entritis helminthes such as *Strongylus* spp., *Trichostrongylus colubriformis* and *H. contortus* (Yacob *et al*., 2002; Terefe *et al*., 2005). Otherwise, the infection of the gastero-intestinal tract with nematodes did not interact with the life cycle of *O. ovis* larvae infection, but oppositely, the infection with *O. ovis* larvae was reduce in the nematode egg excretion and worm burdens (Yacob *et al*., 2002; Terefe *et al*., 2005); these ideas prove that there was no cross-reaction in this study.

Tabouret *et al*. (2003) evaluated the mucosal IgG and IgA responses using ELISA in sheep infected experimentally with *O. ovis* larvae with a low sensitivity and specificity recorded. Some studies investigated the seroprevalence studies using ELISA on *O. ovis*-specific IgG in sheep (Papadopoulos *et al*., 2001; Scala *et al*., 2002, Silva *et al*. 2012) with low sensitivity and specificity in comparison with the present study. This may be attributed to using more sensitive and specific antigens (AC ag).

In the present study, the Dot ELISA gave high sensitivity and specificity results nearly like those by the indirect ELISA. However, the Dot-ELISA has many advantages in comparison with indirect ELISA. The reaction of Dot- ELISA can be read only with a color development which can be evaluated by the eye with no need for a special tool as ELISA reader. Also, several studies examined the validity of antigens dotted onto (NC) which may be stored for over three years at 4°C, or may be valid either for up to ten days or for three months at room temperature or 37°C, so this antigen dotted onto NC membrane could be moved to any country to be used in diagnosis tests, Yamaura *et al*. (2003) and Gupta *et al*. (2008).

Because of the many values of Dot- ELISA, this assay could be used in several diagnoses of many helminthes. It was used in this study on botfly (*O. ovis*) and required only few hours. It is rapid compared to the indirect ELISA which needs two days to be performed.

Gupta *et al*. (2008) explained the advantages of Dot-ELISA confirming that it is a rapid and simple assay and could be performed only using some chemicals and a NC membrane, which means that it could be used in the field. In general, the assay does not require any complicated instruments and the results can be read by the eye. Moreover, Dot- ELISA does not need any special training.

Many parasites were diagnosed using Dot- ELISA including Fascioliasis by Dixit *et al*. (2002), Zimmerman *et al*. (1985); Hydatid cysts by Siavashi *et al*. (2005); Sangaran *et al*. (2017). Toxocariasis by Paller *et al*. (2017); Schistosomiasis by Lakshmanan *et al*. (2016) in addition to many protozoan parasites such as Leishmaniosis, Babesiosis, Amebiasis by Yamaura *et al*. (2003), Toxoplasmosis by Teimouri *et al*. (2018).

5. Conclusion

This study differentiates between two assays to detect the sensitivity and specificity of each test. While the two tests gave nearly similar sensitivity and specificity results, the Dot- ELISA has more advantages as it can be used for diagnosing oestrosis right in the field or in laboratories because of its simple and rapid procedures. On the other hand, ELISA requires special devices with specific laboratory procedures. In addition, its results' development requires two days. To the authors' best knowledge, the Dot- ELISA is the newest serological technique in the diagnosis of oestrosis. Therefore, this study was conducted to evaluate this rapid technique in comparison to the old assay which is frequently used by different scientists. In this study, the two tests confirm the test standards (post-mortem examination), so, the Dot- ELISA (rapid field test) can be easily used in the diagnosis of oestrosis in live animals.

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References

Abo-Shehada MN, Araba B, Mekbela R, Williams D and Torgerson PR. 2000. Age and seasonal variations in the prevalence of *Oestrus ovis* larvae among sheep in northern Jordan. *Prev Vet Med*, **47**: 205–212.

Allaie IM, Wani ZA, Malik AH, Shahardar RA and Zulhuma M. 2016. *Oestrus ovis* larvae in nasal cavity of sheep: a case report. *J Parasit Dis*, **40(4):** 1221–1222.

Alahmed AM. 2000. Seasonal infestation of *Oestrus ovis* larvae in sheep heads in central region of Saudi Arabia. *J Egypt Soc Parasitol*, **30:** 895–901.

Alcaide M, Reina D, Sanchez J, Frontera E and Navarrete I. 2003. Seasonal variations in the larval burden distribution of *Oestrus ovis* in sheep in the southwest of Spain. *Vet Parasitol*, **118**: 235–241.

Amin AR, Morsy TA, Shoukry A and Mazyad SA. 1997. Oestridae head maggots in slaughtered sheep in Cairo abattoirs. *J Egypt Soc Parasitol*. **27**: 855–861.

Angulo-Valadez CE, Scala A, Grisez C, Prevot F, Bergeaud JP , Carta A, Cepeda-Palacios R, Ascencio F, Terefe G, Dorchies P and Jacquiet P. 2008. Specific IgG antibody responses in *Oestrus ovis* L. (Diptera: Oestridae) infected sheep: associations with intensity of infection and larval development. *Vet Parasitol*, **155**: 257–263.

Angulo-Valadez CE, Cepeda-Palacios R, Ascencio F, Jacquiet Ph, Dorchies Ph, Ramı´rez-Ordun J.M and Lopez MA. 2009. IgG antibody response against salivary gland antigens from *Oestrus ovis* L. larvae (Diptera: Oestridae) in experimentally and naturally infected goats. *Vet Parasitol*, **161**: 356–359.

Angulo-Valadez, CE, Ascencio F, Acquiet PJ, Dorchies P and Cepeda-Palacios R. 2011. Sheep and goat immune responses to nose bot infestation: a review. *Med Vet Entomol*. **25**, 117–125.

Attia MM, Ismael E, Saleh NMK. 2019. A sensitive serodiagnos.tic tool for the detection of active infection of zoonotic visceral and nasopharyngeal linguatulosis, *Vet World*, **12(6)**: 883-889.

Bates PG. 1997. The sheep nasal bot fly (*Oestrus ovis*): a forgotten parasite. *Proc. Meet. Sheep Vet Soc*, **21**: 47–52.

Bauer C, Steng G, Prevot F, Dorchies P. 2002. Seroprevalence of *Oestrus ovis* infection in sheep in southwestern Germany. *Vet. Parasitol*. **110**, 137-143.

Bergeaud JP, Duranton C and Dorchies P. 1994. *Oestrus ovis* in Eveyron: results of a study of 1036 sheep heads at the abattoir in Rodez. *Revue de Med Vet*, **145**: 863–866.

Berrag B, Rhalem A, Sahibi H, Tahiri YA, Gray GD and Uilenberg G. 1996. Parasites of goats in the north of Morocco. In: Proceedings of the IFS Workshop, Burkina Faso. *Parasitol. Res. Afr*, 287–306.

Biu AA and Nwosu CO. 1999. Incidence of *Oestrus ovis* infestation in Borno-White Sahel goats in the semi-arid zone of Nigeria. *Vet Res*, **30**: 109-112.

Caracappa S, Rilli S, Zanghi P, Di Marco V, Dorchies P. 2000. Epidemiology of ovine oestrosis (*Oestrus ovis* Linné 1761, Diptera: Oestridae) in Sicily. *Vet Parasitol*, **92(3):** 233-237.

Dixit AK, Yadav SC and Sharma RL. 2002. 28 kDa *Fasciola gigantica* cysteine proteinase in the diagnosis of prepatent ovine fasciolosis. *Vet Parasitol*, **109**:233-247.

Dorchies P, Bergeaud JP, Tabouret G, Duranton C, Prevot F and Jacquiet P. 2000. Prevalence and larval burden of *Oestrus ovis* in sheep and goats in northern Mediterranean region of France. *Vet Parasitol*, **88(3–4)**: 269–273.

Fasih N, Qaiser KN, Bokhari SA, Jamil B and Beg MA. 2014. Human Ophthalmomyiasis externa caused by the sheep botfly Oestrus ovis: a case report from Karachi, Pakistan. *Asian Pac J Trop Biomed*, **4**: 835-7.

Gupta A, Dixit AK and Dixit P. 2008. Dot-ELISA in the diagnosis of parasitic diseases of animals. *J Parasit Dis*, **32(1)**:10–14.

Goddard P, Bates P and Webster KA. 1999. Evaluation of a direct ELISA for the serodiagnosis of *Oestrus ovis* infections in sheep. *Vet Rec*, **144(18)**: 497-501.

Hall M and Wall R. 1995. Myiasis of humans and domestic animals. *Adv Parasitol*, **35**, 257–334.

Harlow E and Lane D. 1988. Antibodies. A laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor.

Innocenti L, Masetti M, Macchioni G and Giorgi F. 1995. Larval salivary gland proteins of the sheep nasal botfly, (*Oestrus ovis*), are major immunogens in infested sheep. *Vet Parasitol*, **60(3-4)**: 273-282.

Jagannath MS, Cozab N, Rahman SA and Honnappa TG. 1989. Factors influencing *Oestrus ovis* infestation in sheep and goats. *Curr Res*, **18**: 118-119.

Karatepe B, Karatepe M and Güler S. 2014. Epidemiology of *Oestrus ovis* L. infestation in sheep in Nigde province, Turkey. *Revue de Medecine Veterinaire*, **165 (7-8)** : 225-230.

Kumar N, Ghosh S and Gupta SC. 2008. Early detection of *Fasciola gigantica* infection in buffaloes by enzyme linked and dot enzyme linked immunosorbent. *Parasitol Res*, **103**: 141–150.

Lakshmanan B, Devada K, Joseph S, Binu MB and Kuttan K. 2016. Efficacy of Dot-ELISA using different antigens in detecting anti-schistosome antibodies among bovines in field conditions. *J Parasit Dis*, **40(1)**: 189–193.

Lowry OH, Rosbrough NJ, Farr AL and Randal RJ. 1951. Protein measurement with Folin phenol reagent. *J Biol Chem*, **193**: 265–275.

Lucientes J, Clavel A, Ferrer-Dufol M, Valles H, Peribanez MA, Gracia-Salinas MJ and Castillo JA. 1997. Short report: One case of nasal human myiasis caused by third stage instar larvae of *Oestrus ovis*. *Am J Trop Med Hyg*, **56(6)**: 608-609.

Martinez S, MorenoT and Becerra C. 1999. Oestrosis. *In*: Cordero del Campillo, M and Rojo Vazquez, FD., (Eds). **Parasitología Veterinaria**. Spain: Mc-Graw Hill, Inter americana, 395-9.

Masoodi M and Hosseini K. 2003. The respiratory and allergic manifestations of human myiasis caused by larvae of the sheep botfly (*Oestrus ovis*): a report of 33 pharyngeal cases from southern Iran. *Ann. Trop. Med. Parasitol*, **97**:75–81.

Ozdal N, Tanrıtanır P, İlhan F and Değer S. 2016. Prevalence of ovine oestrosis (*Oestrus ovis*, Linnaeus, 1761, Diptera: Oestridae) and risk factors in Eastern Turkey. *Veterinarski Arshiv*, **86**: 323-333.

Paller VGV, Besana CM and Valdez IKM .2017. Dot enzyme-linked immunosorbent assay (ELISA) for the detection of *Toxocara* infection using a rat model. *J Parasit Dis*, **41(4)**: 933–939.

Pandey VS and Ouhelli H. 1984. Epidemiology of *O. ovis* infection of sheep in Morocco. *Trop Anim Health Pro*,**16**:246-252.

Pampiglione S, Giannetto S and Virga A. 1997. Persistence of human myasis by *Oestrus ovis* L. (Diptera: Oestridae) among shepherds of the Etnean area (Sicily) for over 150 years. *Parasitol*, **39**: 415–418.

Papadopoulos E, Prevot F, Jacquiet P, Duranton C, Bergeaud JP, Kalaitzakis E and Dorchies P. 2001. Seasonal variation of *Oestrus ovis*-specific antibodies in sheep and goats mixed flocks in Greece. *Vet Parasitol*, **95**: 73–77.

Prosl H and Meyer J. 2003. Ophthalmomyiasis caused by *Oestrus ovis* in a 3-year-old boy. Wien. Klin. Wochenschr,**115**: 76–79.

Reina D, Bonilla F, Martínez-moreno J, Dorchies S, Hernandez S and Navarrete I. 2001. First approach to determining the prevalence of *Oestrus ovis* infestation of sheep in Spain, *In* : Good M, Hall M.J, Losson B, O'Brien D, Pithan K and Sol J (Eds). **Mange and Myiasis in Livestock**, COST Action 833 European Commision, Brussels, Belgium. 145- 150.

Silva BF, Bassetto CC and Amarante AFT. 2012. Immune responses in sheep naturally infected with *Oestrus ovis* (Diptera: Oestridae) and gastrointestinal nematodes. *Vet Parasitol*, **190**: 120– 126.

Sangaran A Bino Sundar ST and Bhaskaran R. 2017. Antigen based detection of cystic echinococcosis in buffaloes using ELISA and Dot-EIASA. *J Parasit Dis*, **41(1):**128–130

Siavashi MR, Taherkhani H, Rezaei KR, Razavi Deligani MR and Assmar M .2005. Comparison of dot-ELISA and sandwich ELISA diagnostic tests in detection of human hydatidosis. *Iran Biomed J*, **9**: 91–94.

Scala A, Solinas G, Citterio CV, Kramer LH, and Genchi C. 2001. Sheep oestrosis (*Oestrus ovis* Linné 1761,Diptera: Oestridae) in Sardinia, Italy. *Vet Parasitol*, **102**: 133–141.

Scala A, Paz-Silva A, Sua´rez JL, Lo´pez C, Dı´az P, Dı´ez-Ban˜os P and Sa´nchez-Andrade Ferna´ndez R. 2002. Chronobiology of *Oestrus ovis* (Diptera: Oestridae) in Sardinia, Italy: guidelines to chemoprophylaxis. *J Med Entomol*, **39**: 652–657.

Tabouret G, Pre´ vot F, Bergeaud JP, Dorchies P and Jacquiet P. 2001. *Oestrus ovis* (Diptera: Oestridae): sheep humoral immune response topurified excreted/secreted salivary gland 28 kDa antigen complex from second and third instar larvae. *Vet Parasitol*, **101**: 53–66.

Tabouret G, Lacroux C, Andreoletti O, Bergeaud JP, Hailu-Tolosa Y, Hoste H, Prevot F, Grisez, C, Dorchies P and Jacquiet P. 2003. Cellular and humoral local immune responses in sheep experimentally infected with *Oestrus ovis* (Diptera: Oestridae). *Vet Res*, **34**: 231–241.

Terefe G, Yacob HT, Grisez C, Prevot F, Dumas E, Bergeaud JP, Dorchies Ph, Hoste H and Jacquiet P. 2005. *Haemonchus contortus* egg excretion and female length reduction in sheep previously infected with *Oestrus ovis* (Diptera: Oestridae) larvae. *Vet Parasitol*, **128**: 271–283.

Trezeguet MA. 1996. Prevalencia de enfermedades en 4000 majadas caprinas en los departamentos Atamisqui, Ojo de Agua, Que brachosy Salavina, provincia de Santiago del epartamentos Atamisqui, Ojo de Agua, Que brachosy Salavina, provincia de Santiago del Estero, Republica de Argentina. *Vet. Argentina*, **13:** 485-489.

Teimouri A, Modarressi M H , Shojaee S, iMohebali M, Zouei N, Rezaian M and Keshavarz H. 2018. Detection of *Toxoplasma*-specific immunoglobulin G in human sera: performance comparison of in house Dot-ELISA with ECLIA and ELISA. *Euro J Clin Microbiol Infect Dis*. doi.org/10.1007/s10096-018-3266-y.

Yamaura H, Araki K, Kikuchi K, Itoda I, Totsuka K and Kobayakawa T. 2003. Evaluation of dot-ELISA for serological diagnosis of amebiasis. *J Infect Chemother*, **9** :25–29.

Yacob HT, Duraton-Grisez C, Prevot F, Bergeaud JP, Bleuart C, Jacquiet Ph, Dorchies Ph and Hoste H. 2002. Experimental concurrent infection of sheep with *Oestrus ovis* and *Trichostrongylus colubriformis*: and related changes in the cellular responses of nasal and digestive mucosae. *Vet Parasitol*, **104**: 307–317.

Yilma JM and Dorchies P. 1991. Epidemiology of *Oestrus ovis* in southwest France. *Vet Parasitol*, **40**: 315–323.

Zimmerman GL, Nelson MJ and Clark CRB. 1985. Diagnosis of ovine fascioliasis by a dot-enzyme linked immunosorbent assay: a rapid diagnostic technique. *Am J Vet Res*, **46 (7):**1513–1515.

Zumpt F. 1965. **Myiasis in Man and Animals in the Old World: A Textbook for Physicians, Veterinarians and Zoologists**. Butterworths, London, 267.

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Beta (β)-Carotene-Induced Effects on the Hepato-Biochemical Parameters in Wistar Rats Fed Dietary Fats

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Abstract

Beta-carotene (βC), a lipid-lowering agent, has been proposed to be a lipid-soluble antioxidant that functions as a precursor of vitamin A. The aim of this study is to evaluate β-carotene- induced effects on hepato-biochemical parameters of Wistar rats exposed to dietary fats. Thirty Wistar rats were divided into six groups. Group A received rat chow, Group B received a high- fat diet, Group C received 300mg/kg body weight (b.wt) of βC, Group D received a high-fat diet for twelve weeks then 300mg/kg b.wt of βC for two weeks, Groups E received 300mg/kg b.wt of βC for two weeks followed by a high-fat diet for twelve weeks while Group F received a high-fat diet for twelve weeks followed by 150mg/kg b.wt of βC for two weeks. At the end, the animals were sacrificed; the blood and liver were collected for analyses while some tissues were fixed for histological studies. The results showed that the level of liver enzymes and bilirubin were increased in Group B compared with the Control (P<0.05). The histological examination showed the liver with fatty accumulation, infiltration by inflammatory cells and fatty vacuolation in the experimental Groups compared to the Control. The results suggest that the β carotene extract can be effective in treating the fatty liver disease.

Keywords: Liver, β-carotene, Dietary fat, Biochemical Parameters, Enzymes, Oxidative stress.

1. Introduction

Dietary fat and its effects on health and diseases have attracted interests for research. Fat is an important source of energy, and facilitates the absorption of fat-soluble dietary components such as vitamins (Javier and Carmen, 2012). An uncontrolled intake of dietary fats could lead to obesity, type 2 diabetes mellitus, dyslipoproteinaemia, hypertension, and metabolic syndrome including the coronary heart disease, strokes and cancer (Wolfram *et al*., 2015). Lipids and insulin play important roles in regulating blood sugar, and altered levels of fat deposits can release triglycerides and free fatty acids into the blood, causing hyperlipidemia (Madubunyi *et al*., 2012). Hyperlipidemia and oxidative stress are major risk factors for atherosclerosis, and are the most important risk factors for cardiovascular diseases (Madubunyi *et al*., 2012; Li *et al*., 2013). Cardiovascular diseases constitute one of the largest public health problems in the world today and are responsible for more than seventeen million deaths annually (WHO, 2005). Moreover, chronic disorders such as cardiovascular diseases, type 2 diabetes mellitus, hyperlipidemia, and obesity are the risk factors for non-alcoholic fatty liver disease (de Alwis and Day, 2009). Non-alcoholic fatty liver disease (NAFLD), regarded as the hepatic manifestation of the metabolic syndrome, currently represents the most common cause of chronic liver diseases (Chalasani *et al*., 2012). NAFLD ranges from simple hepatic fat accumulation (steatosis) to non-alcoholic steatohepatitis (NASH), where fat is accompanied by hepatocyte injury and necroinflammation which pose as increased risk factors for liver cirrhosis and hepatocellular carcinoma (Chalasani *et al*., 2012). Depending on the assessment tools, the prevalence of NAFLD in adults ranges between 20 % and 30 %, reaching up to 46 % in some studies (Bellentani *et al*., 2010). Approximately, 70 % of patients living with NASH also have concurrent dyslipidemia; hence, making treatment with a lipid-lowering medication appear to be a reasonable approach (Williams, *et al.*, 2011; Hyogo *et al*., 2008).

Studies have shown that high-fat diets can easily induce obesity (French and Robinson, 2003), while epidemiological studies have shown that when the average amount of fat in the diet increases, the incidence of obesity also increases (Saris *et al*., 2000). This has led to a worldwide effort to decrease the amount of fat in the human diet. Diets rich in fat not only induce obesity in humans, but also make animals obese (Buettner *et al*., 2007). There is a positive relationship between the level of fat in the diet and body weight in both rats and mice

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(Ghibaudi *et al*., 2002). High-fat diets with fats at 50 % of the total energy in weanling mice, have been used to induce obesity that was called nutritional obesity; the model was later renamed dietary obesity (Fenton & Dowling, 1953). It has been reported that despite the growing problem of obesity, Canadians and Americans are eating less fat than a generation ago (Lissner *et al*., 2000). This shows that the increasing rate of obesity cannot be totally explained by high intakes of fat in the diet, suggesting that the type of fat may also play a role (Moussavi *et al.,* 2008). Some studies have reported that not all fats are obesogenic, and the dietary fatty acid profile, rather than the amount of energy from fat, is an important variable in developing dietary obesity (Ellis *et al*., 2002; Kien *et al*., 2005).

Other factors that may contribute to obesity induced by a diet rich in fat include failure to adjust the oxidation of fat to the extra fat in the diet, increase in adipose tissue lipoprotein lipase activity, increased meal size and decreased meal frequency, overconsumption of energy attributed to high energy density of the diet, orosensory characteristics of fats and poorly satiating properties of the high-fat diets (Buettner *et al*., 2007; Kiess *et al*., 2008). Adipose tissues are considered endocrine organs that secrete cytokines; thus obesity could possibly be regarded as a chronic inflammatory disease (Kiess *et al*., 2008). In the rats fed diets that are high in fat, a linear increase in body fat with an increasing body weight has appeared. The results of the study of Woods et al. (2003) showed that measuring body fat is a more sensitive method for assessing obesity in animals. Tulipano *et al*. (2004) categorized rats that are fed high-fat diets based on their final body weight, with rats in the highest quartile, designated as obesity-prone, and those in the lowest quartile designated as obesity-resistant.

For many decades, medicinal plants have been used to prevent or treat various diseases (Tapiero *et al*., 2004) and some have been used throughout the world for their hypoglycemic, hypolipidemic, or antioxidant activities (Tapiero *et al.,* 2004). Recent studies have shown that carrots have higher concentrations of beta carotene than other dietary sources (Bayerl, 2008). The aim of this study was to evaluate the β-carotene-induced effects on hepato biochemical parameters in the liver of Wistar rats exposed to dietary fats.

2. Materials and Methods

2.1. Preparation of the Extract

Fresh carrots were purchased from Meat Market Abakaliki, Ebonyi State, Nigeria. The carrots were dried under shade for three weeks and were grounded into powder. The pulverized carrots were wrapped in Whatman filter paper and placed in the chamber of Soxhlet extractor. Then 250ml of N-Hexane was added into the Soxhlet flask and placed on a heating mantle. The solvent was heated at 50^0C, the Soxhlet extractor condenses the sample in the filter paper, and the content of the carrots were extracted until a clear solvent started coming out of the extraction chamber. The extract was concentrated using a water bath at 50^0C, and was then stored in the refrigerator.

2.2. Animal Procurement

Ethical approval was arranged and obtained from the Federal University Ndufu Alike Ikwo Ethics and Animal Handling Committee. Thirty Male Wistar rats with average weight of 71.05g were procured from and maintained in the animal house of the Department of Biological Sciences, Federal University Ndufu-Alike Ikwo, Ebonyi State Nigeria. The animals were housed in metal cages, and were given access *ad libitum* to food and water with acclimatization period of two weeks.

2.3. High-Fat Diet Preparation

Cow fat was purchased from Meat Market Abakaliki, Ebonyi State, Nigeria. The fat was dissolved by heating, collected in metal containers and stored in the refrigerator. A high-fat diet was prepared by mixing 60 % of the cow fat and 40 % of normal rat chow as described by Ghibaudi *et al.* (2002) and was then stored in the refrigerator.

2.4. Animal Experimentation

The rats were randomly divided into six groups of five rats in each. Group A (Control) received normal rat chow for fourteen weeks. Group B received a high-fat diet (HFD) daily for fourteen weeks. Group C received 300mg/kg body weight (b.wt) of β-Carotene daily for fourteen weeks. Group D received a high-fat diet daily for twelve weeks followed by 300mg/kg b.wt of β-Carotene daily for two weeks. Group E received 300mg/kg b.wt of β-Carotene daily for two weeks and then HFD daily for twelve weeks. Group F received HFD daily for twelve weeks followed by 150mg/kg b.wt of β-Carotene daily for two weeks. After fourteen weeks of administration, the animals were weighed and humanely sacrificed by cervical dislocation. Blood was collected through cardiac puncture. The animals were dissected, and the liver was harvested, weighed and fixed in 10 % formal saline for a histological examination. The liver weight index in percent (%) was calculated as liver weight over body weight multiplied by 100, as described by Sayed *et al.* (2015). The liver tissues were processed and embedded in paraffin wax. Thin sections of 5 μm thick were made, stained using haematoxylin and eosin (H&E) and examined under a light microscope.

2.5. Biochemical Study

The biochemical parameters studied included serum alanine aminotransferase (ALT), aspartate amino-transferase (AST), Gama Glutamyl Transferase (GGT), alkaline phosphatase (ALP). The activity of ALT was determined using Reitman-Frankel colorimetric method according to the methods of Reitaman and Franke (1957) as modified by Sayed *et al.* (2015) using a Quimica Clinica Applicada (QCA) test kit. ALT activity was measured by monitoring the concentration of pyruvate-hydrazone formed with 2, 4-dinitrophenylhydrazine which is proportional to the concentration at 505nm. The serum was separated by centrifugation at 3600 rpm for fifteen minutes for the determination of serum Gamma glutamyltransferase (GGT) levels using Quimica Clinica Applicada (QCA) commercial test kits according to the Manufacturer's instruction. AST activity was determined by the Reitman-Frankel colorimetric method using a Quimica Clinica Applicada (QCA) test kit. AST activity was measured by monitoring the concentration of

oxaloacetate hydrazone formed with 2, 4–dinitrophenyl hydrazine spectrophotometrically at 505nm. Alkaline phosphatase acts upon the AMP-buffered sodium thymol phthalein monophosphate. Addition of the alkaline reagent stops the enzyme activity and simultaneously develops a blue chromagen which can be measured photometrically at the wavelength of 550nm. Bilirubin content was assayed and estimated according to the Manufacturer's instruction, and the absorbance of the sample against the blank was read at 560nm and calculated.

2.6. Data Analysis

All data were expressed as mean ± SD. The level of homogeneity among the groups was tested using one way Analysis of Variance (ANOVA). Where heterogeneity occurred, the groups were separated using Duncan Multiple Range Test. A value of $P < 0.05$ was considered to indicate a significant difference between groups. Data analysis was done using Statistical Package for Social Sciences (version 20.0).

3. Results

3.1. Liver Weight Index

The results showed a significant increase in the liver weight index of the animals in Group B when compared with those of the Control group (Group A) (P<0.05). There was a significant decrease in the liver index of the animals in Group D when compared with the animals in Group B, but there was a significant increase in the liver index of the animals in Group D when compared with those animals in the Control Group (Group A) (P<0.05). However, there was a significant decrease in the liver index of the animals in Group C when compared with the animals in Group A and other groups (P<0.05) as shown in Figure 1.

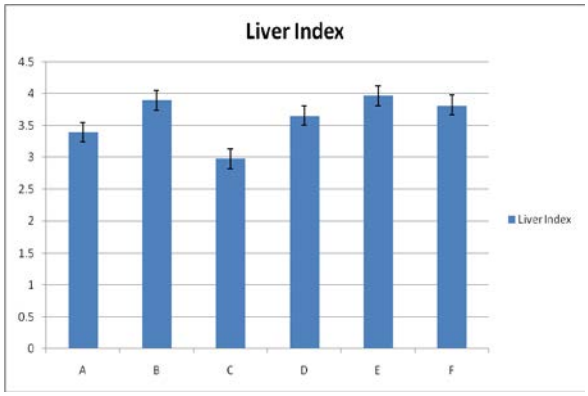

Figure 1. Showing the Liver Index of the animals in different groups

3.2. Effects on Hepatic Function

The serum levels of ALT, ALP, GGT, and Bilirubin were significantly increased (P<0.05) in Group B animals when compared with the animals in the Control (Group A), but there was a significant decrease in the serum level of AST in the animals in Group B when compared with the animals in the Control (Group A) (P<0.05). The results showed a significant decrease in the serum levels of ALP, ALT, AST, GGT, and Bilirubin in the animals in Groups D and F when compared with the animals in Group B (P<0.05) as shown in Table 1.The results also showed that there was no significant difference in the parameters for animals in Group D when compared with the animals in Group E as well. Also, there was no significant difference in the parameters for the animals in group A when compared with the animals in Group C (Table 1).

Table 1: Effect of HFD and Beta Carotene on the hepatic function

GP	ALT (U/L)	AST (U/L)	ALP (U/L)	GGT (U/L)	Total Bilirubin (mg/dl)
A	31.68 ± 0.74*	100.66 ± 0.77	72.56 ± 0.77	11.71 ± 0.84	0.22 ± 0.02*
B	44.68 ± 0.60	83.80 ± 2.39*	79.82 ± 0.98	43.21 ± 2.69**	0.57 ± 0.05**
C	29.72 ± 2.00*	105.86 ± 2.19	64.17± 0.05*	11.62 ± 0.86	0.23 ± 0.00*
D	40.17 ± 0.08	69.67 ± 0.65*	76.67 ± 5.00	22.86 ± 4.73*	0.34 ± 0.02
E	40.72 ± 0.69	68.17 ± 1.50*	74.87 ± 5.14	26.31 ± 1.69*	0.35 ± 0.02
F	42.72 ± 0.72	86.26 ± 8.55	78.17 ± 0.05	41.62 ± 0.72**	0.44 ± 0.02**

Values are expressed as Mean ± SD; N=5, *P<0.05. **

3.3. Effects on Oxidative Stress and Lipid Peroxidation

The results of the present study showed that hepatic superoxide dismutase (SOD) and catalase (CAT) activities in the animals of Group B were significantly decreased when compared with the animals in the Control Group (P<0.05). Malondiadehyde (MDA) levels of the animals in Group B were significantly increased in animals when compared to the animals in the Control Group (P<0.05) (Table 2).

However, the results showed that the oxidative stress and lipid peroxidation markers in the hepatic tissues were restored with a significant increase in SOD and CAT activities, whereas the MDA levels were significantly decreased with the administration of β-Carotene to the animals in Group D when compared with the animals in Group A (P<0.05) as shown in Table 2. The results also showed no significant difference in the SOD and CAT activities and the levels of MDA in the animals in Group C when compared to the animals in Group A as shown in Table 2.

Table 2. Effect of HFD and Beta Carotene on lipid peroxidation and oxidative stress markers

Group	MDA (nmol/mg pro)	SOD (U/mg pro)	CAT (U/mg pro)
A	0.56 ± 0.06**	28.18 ± 2.91**	18.06 ± 0.07**
B	3.77 ± 0.75*	14.00 ± 1.98*	7.17 ± 0.08*
C	0.32 ± 0.12	32.78 ± 3.73	19.81 ± 0.55**
D	1.81 ± 0.42	19.83±0.95*	15.76 ± 2.05**
E	1.76 ± 0.77	20.76 ± 0.63	15.81 ± 0.56**
F	3.12 ± 0.01	18.73 ± 1.99*	11.31 ± 1.26

Values are expressed as Mean ± SD; N=5, *P<0.05 **

3.4. Histological Evaluation

The results showed liver sections from the animals in Group A (Control) with normal hepatic cells radiating from the central vein with well-preserved cytoplasm and nucleus as shown in Figure 2A. The histology of the liver

of animals in Group B showed hepatic tissues with steatosis due to the presence of fat accumulations resulting in hepatocytes vacuolation as in Figure 2B. Meanwhile, the liver section of the animals in Group C showed normal hepatic architecture (Figure 2C). The results showed that the degree of hepatic injury including steatosis, hepatocytes with cytoplasmic vacuolation and lobular inflammation were to a lesser degree in the animals in Groups D and E as shown in Figures 2D and 2E. Thus steatosis with inflammation and hepatocytes with cytoplasmic vacuolation were attenuated with beta carotene for two weeks. The results also showed that beta carotene (Group F) had been shown to reduce the level of the distortions to the hepatic tissues as shown in Figure 2F.

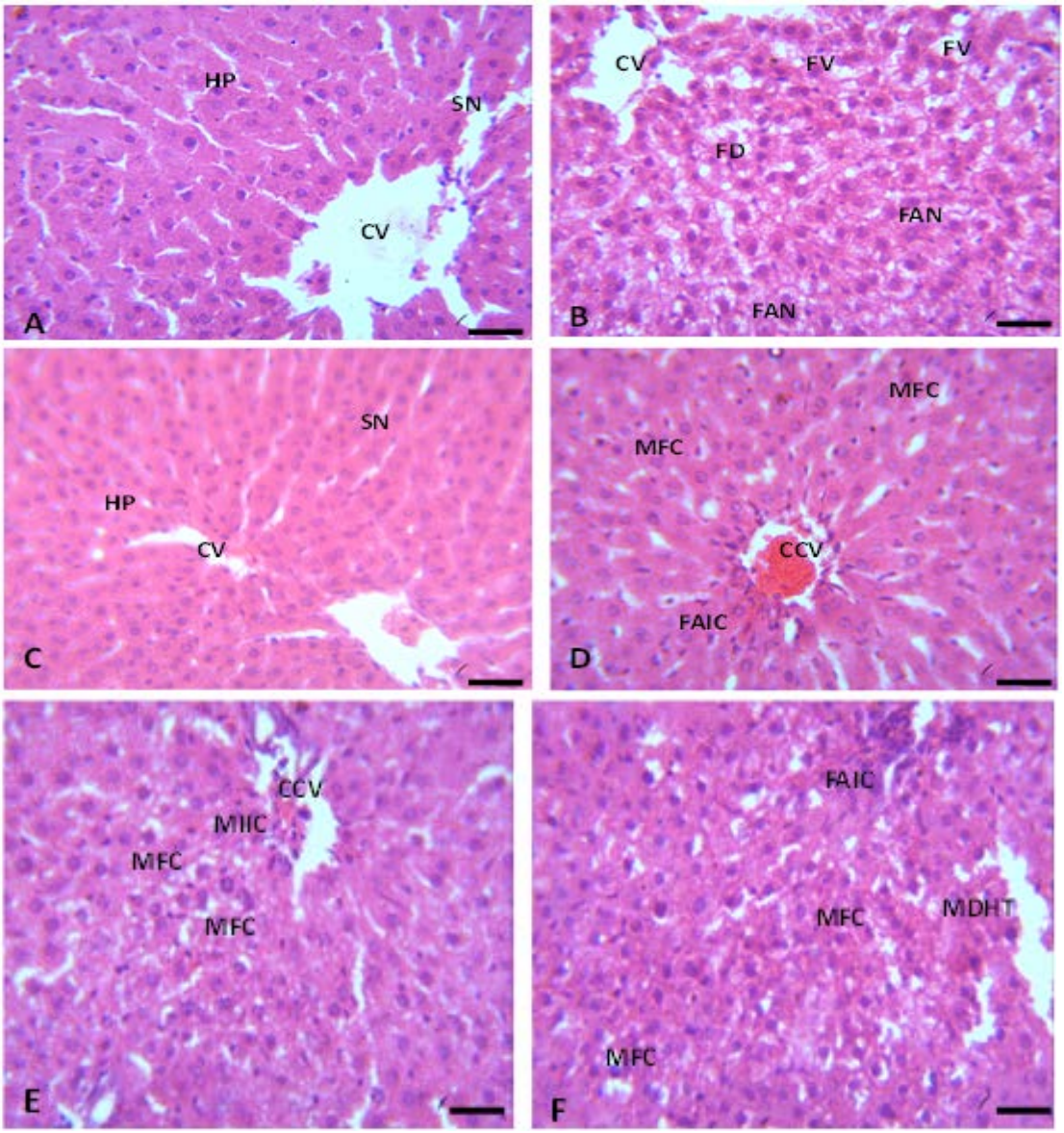

Figure 2. (A) Section of the Liver of Group A (Control) animals showing normal hepatic architecture with central vein (CV) sinusoids (SN) and hepatocytes (H); (B) Section of the Liver of Group B animals showing fatty changes (FC), fatty necrosis, and distortion of hepatic tissue; (C) A section of the liver of Group C showing normal hepatic architecture with central vein (CV), hepatocytes (H), and Sinusoids (S); (D): Section of the liver of Group D showing mild fatty changes (MFC), focal aggregate of inflammatory cells (FAIC), and congestion of central vein (CCV); (E) A Section of the liver of Group E showing mild fatty changes (MFC) and moderate infiltration of inflammatory cells (MIIC); (F) Sections of the liver of Group F showing, moderate regeneration with mild fatty change (MFC), focal aggregate of inflammatory cells (FAIC), and mild distortion of hepatic tissue (MDHT). Stain: H & E; Magnification: x400. Scale Bar: 1mm=5µm

4. Discussion

The results of this study showed an increase in the liver weight index in animals fed with dietary fats alone for twelve weeks, while the liver weight index was significantly reduced with the administration of beta-carotene for two weeks. This showed that β-Carotene can be active in reducing the fat deposits in the liver, and thereby decreasing the hepatic index.

The present study has shown that β-Carotene administration resulted in a significant decrease in liver enzymes when compared with the animals fed with dietary fats. This is in agreement with the study of Vardi *et al.* (2010) who reported that β-Carotene given for twenty-one days before the methotrexate application provided significant protection from hepatotoxicity resulting from

methotrexate intoxication. Chung *et al*. (2009) had reported that in hyperlipidemic patients, there was a clinically significant elevation in the levels of AST/ALT, and this was in agreement with the results from the present study. It has been shown that visceral fats release free fatty acids which are transported to the liver by the portal, vein and may contribute to hepatic steatosis, the production of triglyceride rich in very low density lipoprotein (VLDL). The elevated β-oxidation can contribute to the significant elevation of the liver AST/ALT (Chung *et al*., 2009).

However, the elevated serum liver enzymes namely AST, ALT, ALP, GGT, and Bilirubin levels, were reversed upon treatment with beta-carotene, and this was in accordance with Jensen (2008), who observed that most of the carotenoids appeared to be inversely correlated to the fat mass, suggesting that in obesity, carotenoids are sequestered in adipose tissues thereby decreasing their plasma concentrations. Moreover, van Helden *et al*. (2011) had demonstrated that the anti-obesity effects of β-carotene were linked to its pro-vitamin A effects which means that β-carotene exerts its effects by functioning as a precursor of vitamin A. Thus β-carotene functions as a lipid-lowering agent and a lipid-soluble antioxidant. The results also showed that the administration of beta-carotene before feeding the rats with dietary fats helped to protect the liver against damage by maintaining the levels of the serum liver enzymes, lipid profile, and adipocytokine markers (Okechukwu *et al.*, 2018).

The observed elevation in all the liver enzymes in animals fed with dietary fats was also in agreement with AL-Dosari *et al*. (2011), who had reported that there were increased plasma activities of AST, ALT, ALP and GGT in the fat diet-fed rats. ALT and AST are biomarkers in the diagnosis of hepatic damage because they are released into the circulation after hepatocellular damage (Naik and Panda, 2007). ALT and AST are liberated into the blood whenever liver cells are damaged, and thus resulting in increased plasma enzyme levels, and has been shown to be a very sensitive index of liver damage (Edwards *et al*., 2008; Crook, 2012). Although none of these enzymes are specific to the liver, but ALT occurs in much higher concentration in the liver than elsewhere. Therefore, the increased serum ALT level in the present study, more specifically, reflects hepatic cellular damage (Edwards *et al*., 2008; Crook, 2012; Ekandem and Peter, 2015).

The results obtained from the present study revealed that intake of dietary fats at 60 % fat levels for twelve weeks have been shown to have damaging effects on the liver of Wistar rats with the distortion of the liver cellular architecture, dilation of the hepatocytes with congestion of the central vein, dilation of the sinusoidal spaces, and infiltration of the inflammatory cells with cytoplasmic ground glass appearance of the hepatic tissues. The results showed that the hepatic injury was ameliorated by the administration of beta-carotene for two weeks. This was in agreement with the report given by Sayed *et al*. (2015). Meanwhile, the result also revealed that there have been dose-dependent effects of beta-carotene on the liver, as the administration of 300mg/kg body weight of beta-carotene showed a better treatment outcome to the hepatic injury caused by the fat diets than the 150mg/kg body weight of beta-carotene. However, the pre-treatment of beat-carotene to the Wistar rats before exposure to dietary fats has a protective effect against hepatic damage. The major drawback of the present research is the small sample size of five animals used in a group which stand as a major and important limitation to the study. Therefore, further larger scale studies are needed to verify and solidify the findings.

5. Conclusion

The consumption of β Carotene-rich foods, fruits and vegetables have been shown to have enhancement effects in modulating the hepatic functions and histological structures related to liver damages especially when it relates to the nonalcoholic fatty liver disease. This may be due to their synergistic anti-oxidative, anti-inflammatory, and lipid-lowering effects in the body while the pre-treatment of beta-carotene before a dietary fat intake protected the liver from the manifestation of the hepatic injuries in the Wistar rats, βeta carotene could be recommended as a remedy for the treatment of the nonalcoholic fatty liver disease, especially that resulting from the high-fat diet consumption.

References

AL-Dosari M, ALqasoumi S, Ahmad M, AL-Yahya M, Ansari MN and Rafatullah S. 2011. Effect of *Beta vulgaris* L. on cholesterol rich diet-induced hypercholesterolemia in rat, *Farmacia*. **59**:669–678.

Bayerl C. 2008. Beta-carotene in dermatology: Does it help, *Acta Dermatovenerol Alp Panonica Adriat.***17:** 10-162.

Bellentani S, Scaglioni F, Marino M and Bedogni G. 2010. Epidemiology of non-alcoholic fatty liver disease. *Digestive Dis*., **28**:155–161.

Buettner R, Scholmerich J and Bollheimer LC.2007. High-Fat Diets: Modelling the metabolic disorders in human obesity in rodents. *Obesity*. **15**: 798-808.

Chalasani, N., Younossi, Z., Lavine, JE., Diehl, AM., Brunt, EM., Cusi, K., Charlton, M and Sanyal, AJ. 2012. The Diagnosis and Management of Non-Alcoholic Fatty Liver Disease: Practice Guideline by the American Association for the Study of Liver Diseases, *Am College of Gastroenterol*. *Am Gastroenterol Asso*.

Chung, HK, Kang, B., Lee, JH, Shim, JY, Park, S, Lee, SH, Park, YK, Park, E, Chung, N. and Shin, MJ. 2009. Increased arterial stiffness is associated with reduced plasma levels of beta-carotene in treated hypertensive patients with type 2 diabetes mellitus. *Nutrition, Metabol Cardiovascular Dis.*, **6**: e9–e11.

Crook, MA. 2012. **Clinical Boichemistry and Metabolic Medicine**. 8th ed. Edwaed Arnold Publishers, London. 254-255.

de Alwis, N.M. and Day, C.P. 2009. Non-alcoholic fatty liver disease: the mist gradually clears, *J Hepatol.*, **48 (1)**: S104-12.

Edwards, CRW, Bouchier, IAD, Haslett, C. and Chilvers, EE. 2008. **Diabetes mellitus in Davidson's Principle and Practice of Medicine** (10th Edition). Churchill Livingstone, London. Pp. 724-774.

Ekandem, JG and Peter, AI. 2015. Histological and biochemical effects of chronic chloramphenicol administration on the liver of Wistar rats. *J Anatomical Sci.*, **6(2)**: 85

Ellis J, Lake A and Hoover-Plow J. 2002. Monounsaturated canola oil reduces fat deposition in growing female rats fed a high or low fat diet. *Nutr. Res*. **22**: 609–621.

Fenton PF and Dowling MT.1953. Studies on obesity. Nutritional obesity in mice. *J Nutr*.**49**: 319–331.

French S and Robinson T. 2003. Fats and food intake. *Curr Opin Clin Nutr Metab Care*.**6**: 629–634.

Ghibaudi L, Cook J, Farley C, van Heek M and Hwa JJ. 2002. Fat intake affects adiposity, comorbidity factors, and energy metabolism of sprague-dawley rats. *Obes Res.***10**:956–63.

Hyogo H, Tazuma S, Arihiro K, Iwamoto K, Nabeshima Y, Inoue M, Ishitobi T, Noonaka M and Chayama K. 2008. Efficacy of atorvastatin for the treatment of nonalcoholic steatohepatitis with dyslipidemia. *Metabolism*. **57 (12):**1711–1718.

Javier A and Carmen PR.2012. Recommended dietary reference intakes, nutritional goals and dietary guidelines for fat and fatty acids: a systematic review, *Br J Nutr*., **107**: 8-22.

Jensen MD. 2008. Role of body fat distribution and the metabolic complications of obesity, *J Clin Endocrinol Metabolism*. **93**:S57–S63.

Kien CL, Bunn JY and Ugrasbul F. 2005. Increasing dietary palmitic acid decreases fat oxidation and daily energy expenditure. *Am J Clin Nutr*.**82**: 320–326.

Kiess W, Petzold S and Topfer M. 2008. Adipocytes and adipose tissue. *Best Pract Res Clin Endocrinol Metab*.**22**: 135–153.

Li C, Zhang W, Zhou F, Chen C, Li Y, Liu L, Pei F, Luo H, Hu Z, Cai J and Zeng C. 2013. Cholesteryl ester transfer protein inhibitors in the treatment of dyslipidemia: a systematic review and meta-analysis, *PLoS One*. **8**:e77049.

Lissner L, Heitmann BL & Bengtsson C.2000. Population studies of diet and obesity. *Br J Nutr*.**83**: S21–S24.

Madubunyi II, Onoja SO, Asuzu IU. 2012. *In vitro* Antioxidant and *in vivo* antidiabetic potential of the methanolic extract of *Ficus glumosa* Del (Moraceae) Stem bark in alloxan-induced diabetic mice. *Comp Clin Pathol*.**21**: 389-394.

Moussavi N, Gavino V and Receveur O. 2008. Could the quality of dietary fat, and not just its quantity, be related to risk of obesity? *Obesity*.**16**: 7–15.

Naik SR and Panda VS. 2007. Antioxidant and hepatoprotective effects of *Ginkgo biloba* phytosomes in carbon tetrachloride-induced liver injury in rodents. *Liver Inter.*, 27:393–399.

Okechukwu, NG, Nweke, BO, Uchewa OO, Ezemagu, KU and Ibegbu, AO. 2018. Tumor necrosis factor alpha (TNF-α) and plasma glucose level changes due to dietary fat and beta carotene in Wistar Rats. *J Advancs in Medi. Medical Res*., **26(12**): 1-7.

Reitaman S and Franke lS. 1957. A colorimetric method for the determination of serum glutamate-oxaloacetic acid and glutamate-pyruvic acid transaminases. *Am J Clin Pathol.,* **28**: 56-63.

Saris WHM, Astrup A and Prentice AM. 2000. Randomized controlled trial of changes in dietary carbohydrate/fat ratio and simple vs complex carbohydrates on body weight and blood lipids: The CARMEN study. The Carbohydrate Ratio Management in European National diets. *Int J Obes.* **24**: 1310–1318.

Sayed HS, Naglaa ME and Fatma AE. 2015. Effects of Resuvastatin and/or β-carotene on nonalcoholic fatty liver disease in rats. *Res Pharmaceutical Sci*., **10(4):** 275-287.

Tapiero, H., Townsend, DM and Tew, K.D. 2994. The role of carotenoids in the prevention of human pathologies. *Biomed. Pharmacother*. **58**: 100-110.

Tulipano G, Vergoni AV and Soldi D. 2004. Characterization of the resistance to the anorectic and endocrine effects of leptin in obesity-prone and obesity resistant rats fed a high-fat diet. *J Endocrinol*.**183**: 289–298.

Van Helden YG, Godschalk RW, Swarts HJ, Hollman PC, van Schooten FJ and Keijer J. 2011. Beta-carotene affects gene expression in lungs of male and female Bcmo1 (-/-) mice in opposite directions. *Cell Mol Life Sci*.**68**:489–504.

Vardi N, Parlakpinar H, Cetin A, Erdogan A and Cetin Ozturk I. 2010. Protective effect of beta-carotene on methotrexate-induced oxidative liver damage, *Toxicologic Pathol.*,.**38**:592–597.

Williams CD, Stengel J, Asike MI, Torres DM, Shaw J, Contreras M. 2011. Prevalence of nonalcoholic fatty liver disease and non-alcoholic steatohepatitis among a largely middle-aged population utilizing ultrasound and liver biopsy: a prospective study. *Gastroenterol*. **140**:124–131.

Wolfram G, Bechthold A, Boeing H, Ellinger S, Hauner H, Kroke A, Leschik-Bronnet E, Linseisen J, Lorkowski S, Schulze M, Stehle P and Dinter J. 2015. Evidence-based guideline of the German Nutrition Society: Fat intake and prevention of selected nutrition-related diseases. *Ann Nutr Metab*. **67**(3): 141-204.

Woods SC, Seeley RJ and Rushing PA.2003. A controlled high-fat diet induces an obese syndrome in rats. *J Nutr.***133**: 1081–1087.

World Health Organization (WHO).2005. Obesity and overweight.*http://www.who.int/mediacentre/factsheets/fs311/en/index.* Geneva. *Accessed on 20th August, 2016.*

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Phylogenetic Relationship among Some species of the Genera *Lens*, *Vicia*, *Lathyrus* and *Pisum* (Leguminosae) in Palestine

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Abstract

Leguminosae or Fabaceae is the third largest flowering plant family. It is economically important for food production and soil fertility. The molecular phylogenetic analysis of closely related species of Leguminosae family: *Lens culinaris, Vicia sativa, V. palaestina, V. peregrina, V. faba, V. narbonensis, Lathyrus aphaca, Pisum fulvum* and *P. sativum* aids in the discrimination among these closely related species. In this study, 18S and 28S as universal primers were used for amplifying and sequencing the internal transcribed spacer (ITS) region of the studied species. This was conducted on around two-five plant individual samples of each one of the species under investigation. The phylogenetic tree construction was carried out using Unweighted Pair Group method. The phylogenetic analysis among the studied species revealed that *V. peregrina, V. faba, V. narbonensis, V. palaestina, Lens culinaris,* and *V. sativa* were grouped into one clade (clade I). However, *V. sativa* occupied the farthest position in clade I, and was separated from other species of *Vicia* genus. *P. sativum, P. fulvum* and *Lathyrus aphaca* were grouped into another clade (clade II). On the other hand, *Lens culinaris* occupied a position within the core of *Vicia* near to *V. palaestina*. Accordingly, it is recommended to transfer *Lens culinaris* into *Vicia* genus based on its morphological and molecular characterization. Therefore, the new classification of *Lens culinaris* is recommended to be *Vicia culinaris.*

Keywords: Fabaceae, Internal Transcribed Spacer, (ITS), *Lathyrus*, *Lens*, Palestine, *Pisum*, rDNA, *Vicia.*

1. Introduction

The genetic diversity via the evolutionary process is very important for the survival of the species as it assists the species to adapt to environmental changes through the natural selection process and reduces their extinction risk (Grassi *et al*., 2006).

Legumes are flowering plants which belong to the Fabaceae (Leguminosae**)** family. They are different in size and in habit varying from herbaceous to woody plants, which are widely distributed worldwide. Legumes are used as an important source of food for humans and animals; in addition to their synthesis of many secondary compounds for medical principles, coloring, etc. (Andrea, 2011).

Each genus of the four Fabaceae genera (*Lens, Vicia*, *Lathyrus* and *Pisum*) has at least one unique character, which aids in the discrimination among them. Although legumes' classification depends on the morphological characters, many conflicts are encountered in this process (Andrea, 2011). Moreover, morphological continuum is observed, in particular between *Lens* and *Vicia*. Hence, *Lens* is a *Vicia* with a *Lathyrus* style characters. Moreover, *V. sativa* var. *platysperma* and *V. lunata* have an intermediate form between *Vicia* and *Lens* (Erskine *et al*., 2009). Therefore, molecular tools may provide better classification and identification discrimination.

The rDNA (ribosomal DNA) genes are specific genes of the nuclear genome that are used for genetic diversity (Zhang *et al*., 1990). The high degree of variation of the internal transcribed spacers (ITS), even between closely-related species, has been helpful to many biodiversity topic studies (Nickrent and Patrick, 1998; Penteado *et al*., 1996; Polanco and Perez, 1995; 1997).

Morphological and cytological studies of *Lens montbretti* recommended its transfer from the genus *Lens* to the genus *Vicia* classifying it as *Vicia montbretti* (Ladizinsky and Sakar, 1982). Moreover, the molecular analysis of *Lens montbretti* and *V. montbretti* helped in the reclassification of *Lens montbretti* in the genus *Vicia*, in spite of its lentoid calyx, style, and flattened seed characteristics (Mayer and Bagga, 2002). Similarly, the prominence of molecular information correlation with the morphological information was shown by the fact that *P. sativum* is sister to the monophyletic *Lathyrus* species. Moreover, *Lens* species created a clade near to the *Vicia* species, indicating that *Lens* is a close genus to *Vicia* (Steele and Wojciechowski, 2003).

Therefore, the Phylogenetic relationship among different species (*Lens culinaris, V. sativa, V. palaestina, V. peregrina, V. faba, V. narbonensis, Lathyrus aphaca,*

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Pisum fulvum and *P. sativum*) was studied. In addition, the taxonomic classification conflict of *Lens culinaris* and its phylogenetic relationship to the morphological closely related species *Vicia palaestina* was investigated. Data considering the classifications of these plant species based on molecular techniques and sequence information seem to be the first of their kind in Palestine.

2. Materials and Methods

2.1. Plant Material Collection

Two to five fresh plant specimens of the species under investigation were collected from different localities in Palestine via intensive field trips during the period of study (December-April, 2014). Voucher herbarium specimens were deposited at herbarium of the Department of Biology and Biotechnology, Faculty of Science, An-Najah National University (Table 1).

2.2. Taxonomical Analysis and Identification of the Collected Plant Species

The collected plant species of the legume genera under investigation were classified and identified relying on their morphological characters according to Flora Palaestina (Zohary 1987).

Table 1. Scientific names of the studied species with their: common names, wild or cultivated states, the locations from where they were collected, and voucher numbers.

Scientific name	Common name	Wild vs.Cultivated	Place	Accession No.	Voucher numbers
Lathyrus aphaca L.	Yellow pea, Yellow vetch	Wild	Tubas	KJ864924	1606a
		Wild	Tubas	KJ864925	1606b
P. fulvum Sm.	Tawny pea	Wild	Salfit	KJ864933	1598
		Wild	Taluza	KJ864934	1607a
		Wild	Taluza	KJ864935	1607b
		Wild	Taluza	KJ864936	1607d
P. sativum L.	Garden pea	Cultivated	Aqqaba	KJ864943	1579
		Cultivated	Yasid	KJ864945	1580
		Cultivated	Qalqilia	KJ864942	1584
		Cultivated	Maithaloun	KJ864944	1586
		Cultivated	Salfit	KJ864946	1609
V. palaestina Boiss.	Palestine vetch	Wild	Beit-Wazan	KJ864940	1603
		Wild	Beit-Leed	KJ864937	1610a
		Wild	Beit-Leed	KJ864941	1610b
		Wild	Al-Ameryah	KJ864938	1611
		Wild	Tubas	KJ864939	1570
V. peregrine L.	Rambling vetch	Wild	Salfit	KJ864952	1600a
		Wild	Salfit	KJ864955	1600b
		Wild	Beit-Wazan	KJ864953	1613a
		Wild	Beit-Wazan	KJ864956	1613b
		Wild	Al-Ameryah	KJ864954	1614
V. sativa L.	Common vetch	Cultivated	Yamun	KJ864947	1601a
		Cultivated	Yamun	KJ864950	1601b
		Cultivated	Tamun	KJ864948	1602a
		Cultivated	Tamun	KJ864951	1602b
		Wild	Beit-Wazan	KJ864949	1612
V. faba L.	Broad bean, fava bean	Cultivated	Maithaloun	KJ864957	1578
		Cultivated	Salfit	KJ864958	1582
V. narbonensis L.	Narbon bean	Wild	Tubas	KJ864959	1571a
		Wild	Tubas	KJ864960	1571b
Lens culinaris Medik.	Lentil	Cultivated	Yasid	KJ864928	1581a
		Cultivated	Yasid	KJ864929	1581b
		Cultivated	Qalqilia	KJ864930	1585a
		Cultivated	Qalqelia	KJ864931	1585b
		Cultivated	Maithaloun	KJ864932	1599

2.3. Genomic DNA Extraction

The total genomic DNA from previously frozen leaf samples of the studied plant species was extracted. *Salvia dominica* (Labiatae) was included as an outgroup. The frozen leaf samples were ground into a fine powder using mortar and pestle in the presence of liquid nitrogen. After that, the genomic DNA was extracted from a total of 0.1 g of the leaf tissue powder using PureLink™ Plant Total DNA Purification Kit (Invitrogen, USA), following the manufacturers protocol for the isolation of total genomic DNA.

2.4. PCR Amplification and Gel Electrophoresis

The nuclear ribosomal DNA encompassing the ITS regions (ITS-1 spacer, 5.8S rDNA and ITS-2 spacer) using

universal primers. Here the primer sequences were 5′-CCT TMT CAT YTA GAG GAA GGA G-3′ for 18S and 5′-CCG CTT ATT KAT ATG CTT AAA-3′ for 28S. The PCR reaction mix with a final volume of 25 µL, was performed with 12.5 µL of PCR premix (ReadyMix^{TM}Taq PCR Reaction Mix with $MgCl_2$, Sigma, USA), 0.4 µM of each primer and 2 µL of DNA template. The amplification was carried out using the thermal cycler (Mastercycler personal, Eppendorf, Germany) according to the following thermal conditions: initial denaturation for three minutes at 94 ºC was followed by thirty-five cycles of denaturation at 94 ºC for forty-five seconds, annealing at 56 ºC for one minute and extension at 72 ºC for two minutes, with a final extension step at 72 ºC for five minutes. The PCR products were resolved by electrophoresis through 1.5 % agarose gel to determine the size of the amplified fragment after ethidium bromide staining (Muir *et al*., 2001).

2.5. DNA Cleaning and Sequencing

The obtained PCR products were cleaned with ChargeSwitch®-Pro PCR Clean-Up Kit (Invitrogen, USA), following the manufacturer's protocol PCR product clean up. The DNA PCR products were sequenced by dideoxynucleotide chain termination method using the 3130 Genetic Analyzer (Applied Biosystems®, USA), Bethlehem University, Bethlehem, Palestine. The sequencing PCR reaction was performed with 18S and 28S primers used singly in forward and reverse reactions and BigDye® Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems®, USA). Sequences were further submitted for accession numbers in primary bioinformatics web servers.

2.6. Sequence Alignment and Phylogenetic Analysis

The sequences of ITS region of the examined nine Leguminosae species were compared with previously available sequences in NCBI (National Center for Biotechnology Information) using BLAST (Basic Local Alignment Search Tool) system. Multiple alignments were done using ClustalW of the computer program CLC Main Workbench software (version 5.6.1, 2009, CLC bio, Aarhus, Denmark). Pairwise distances were generated using the Kimura 2-parameter method. Phylogenetic analyses were based on alignments obtained from ClustalW of a 600 bp sequence. After that, a phylogenetic tree was constructed using the program Unweighted Pair Group Method with Arithmetic Mean (UPGMA) in the same software.

The robustness of the groupings in the UPGMA analysis was assessed with 1000 bootstrap (bs) re-samplings. Reference sequences were retrieved from GenBank and were used for the phylogenetic analyses. *Salvia dominica* was used as an outgroup.

3. Results

3.1. Description of the Studied Plant Species

3.1.1. Lathyrus aphaca L.

Annual, glaucescent, subglabrous, 10-75 cm. Stems: usually erect or ascending, branched mainly at base, flexuous, angular. Leaves: abortive, reduced to stipules and tendrils; stipules: 0.5-4 cm., sessile, simple, leaf-like, ovate, sagitate-hastate or truncate at base, apiculate; tendrils: 1-6 cm., simple, filiform. Peduncles: as long as tendrils and longer than stipules, muticous or short-aristate. Racemes axillary, 1(-2)-flowered. Pedicles: as long as the calyx tube, erect or slightly curved, often hairy. Flowers: 1-1.5 cm. Calyx: about 1 cm.; teeth much longer than tube, almost equal, lanceolate, acute. Corolla: longer than calyx, yellow; standard longer than the wings and the incurved, whitish and pink-veined keel. Pod 3(-4-7)-seeded, 2-3 x 0.4-0.6 cm., erect, compressed, oblong-linear, sometimes falcate, slightly torulose, beaked, reticulately veined. Seeds: 2-4 mm., subglobular, brown-black, smooth. Fl. February-April.

3.1.2. Pisum fulvum Sm.

Annual, glabrous, 15-70 cm. Stems: ascending or procumbent, rarely erect, slender. Leaves: 3-12 cm., spreading; stipules: 1-4 cm., ovate, semicordate, dentate or incised all around or up to middle; leaflets 1 (-2)-paired, 1.5-2.5 x 1-1.5 cm., ovate, mostly dentate. Racemes 1 (-2-3)-flowered, with peduncles longer than stipules. Flowers: about 1 cm. or less. Corolla: rusty-yellow or riddish-brown, pale in subterranean flowers; standard broad, ovate to orbicular, retuse to two-lobed. Pod 2.5-3 (-4) x 0.7-1 cm., short-beaked, net-veined. Seeds: about 4 mm., black, velvety, punctulate. Fl. February-April.

3.1.3. Pisum sativum Tackholm

Annual, glabrous, 40-150 cm. Stems: angular or roundish, hollow, covered with a waxy bloom. Leaves: 6-15 cm., spreading; stipules: about 8 cm., ovate, semisagittate; leaflets (0-) 1-2 (-3)-paired, 1-5 x 1-4 cm., broad, elliptic to oblong, entire to coarsely-toothed. Racemes 1-4-flowered, with peduncles shorter than stipules. Flowers: about 3 cm. Corolla: white to pink or purple; standard broad, ovate to orbicular, retuse to two-lobed. Pod 4-15 x 1.5-2.5 cm., short-beaked, net-veined. Seeds: about 5 mm., whitish, gray, green or brownish, smooth or wrinkled punctulate. Fl. February-April.

3.1.4. Vicia palaestina Boiss.

Annual, sparingly appressed-hairy, 15-80 cm. or more. Stems: climbing, simple to branched, slender. Leaves: 2-7.5 cm., subglabrous to pubescent; stipules: 2-4 mm., semihastate, those of the uppermost leaves lanceolate to oblanceolate; tendrils often branched; leaflets (5-) 6-10-paired, 0.5-3 x 0.05-0.3 (-0.5) cm., subsessile, narrowly linear to narrowly oblanceolate, acute to obtuse, mucronulate. Peduncles: long but shorter than subtending leaves, muticous. Racemes (2-) 3-8 (-9)-flowered, generally one-sided. Pedicels: about as long as calyx, pubescent. Flowers: (5-) 6-9 mm., deflexed. Calyx: about 2 mm., somewhat hairy; rim of tube: slightly oblique; teeth: a little shorter than tube, the lower teeth: longer, lanceolate-triangular. Corolla: about three times as long as calyx; standard longer than wings, blue, slightly retuse at apex; wings: white-blue or cream-blue; keel dark blue at apex. Style: subcompressed, hairy at apex. Pod: (1.3-) 2-2.5 x (0.4-) 0.5-0.8 cm., stipitate, 1-4-seeded, compressed, rhombic-elliptical to oblong, more or less torulose, short-beaked, glabrous, somewhat net-veined. Seeds: 3-6 mm., globular to compressed-ovoid, brown to blackish-brown, smooth; hilum short, linear. F1. February-May.

3.1.5. Vicia peregrina L.

Annual, appressed-puberulent or pubescent, 15-60 (-75) cm. Stems: procumbent to erect, usually branched,

angular. Leaves: 1.5-6.5 cm.; stipules: 2-4 mm., narrow, semihastate or semisagittate, free portion subulate or lanceolate, pilose; tendrils: simple to branched; leaflets: 2-) 3-7-paired, 1-3 x 0.1-0.6 cm., subsessile, narrowly linear to oblanceolate, tapering at base, retuse, rarely acuminate, mucronulate. Racemes: axillary, mostly 1-flowered. Pedicels: about as long as to a little longer than calyx, hairy. Flowers: 1.1-2 cm. Calyx: 6-7 mm., slightly gibbous, with an oblique limb; teeth: almost as long as tube, the upper teeth shorter, connivent, lanceolate, acuminate. Corolla: about twice as long as clayx, purple or blue-violet, paler at base, sometimes white; standard longer than wings, notched. Style: hairy at apex. Pod: 2-4 x 0.4-1.2 cm., short-stipitate, 3-7-seeded, deflexed, more or less compressed, oblong-linear, shorter-beaked, appressed-hairy to subglabrous, sometimes with violet-purple spots. Seeds: about 4 mm., subglobular, sometimes subangular, mostly dark brown or mottled with black; hilum oblong, dark. Fl. February-May.

3.1.6. Vicia sativa L.

Annual, hairy to subglabrous, 20-80 cm. Stems: erect to procumbent, branching from base. Leaves: 3-11 cm.; stipules varying in length, semihastate, dentate, usually with a purple nectary spot beneath; tendrils: usually branched; leaflets: 4-10-paired, varying in size and shape, 1-3 x 0.4-1 cm., linear or lanceolate to oblong or obovate, sometimes elliptical, obcordate or cuneate, acutish or obtuse to truncate or retuse, mucronate, mostly entire. Racemes: axillary, almost sessile, 1-3-flowered. Flowers 1-3 cm., short-pedicelled. Calyx 1-3 cm., campanulate, hairy, rim of calyx tube even (not oblique); calyx teeth 0.3-1 x 0.1-0.2 cm., subequal, linear-subulate or lanceolate, acute-mucronate. Corolla: one and a half to two and a half times as long as calyx, sometimes two-coloured; standard 0.7-1.3 cm., broad, obvate-orbicular, notched, whitish-pink to purplish-violet, claw about as long as limb; wings shorter than standard, bluish-pink to purplish-violet; keel shorter than wings, paler, usually darker at apex. Pod: 3-6.5 x 0.4-1 cm., 2-10-seeded, pods compressed to turgid, linear, torulose or not, more or less pubescent, net-veined, yellowish to brown or black, rarely whitish. Seeds: 3-6 mm., rarely larger, subglobular, sometimes compressed, plain or variegated, greenish-grey or brown-yellow or black; hilum short, linear. Fl. (February-) March-May (-June).

3.1.7. Vicia faba L.

Annual, glabrous, 30-160 cm. Stems: erect, unbranched. Leaves: 6-12 cm.; stipules 1-2 mm., sagittate, base toothed; tendrils 0 or rudimentary; leaflets: 2-6-paired, 5-8.5 x 2.2-3.5 cm., ovate to elliptic, obtuse to acute. Racemes: axillary, subsessile, mostly 2-6-flowered. Floweres: 2-3 cm., dull white. Calyx: 7-15 mm., campanulate, unequal toothed; calyx teeth: 0.5 x 0.3 mm., Corolla: about twice as long as clayx, white wings with dark blotches; standard 1.5 cm., broad, wings: shorter than standard, keel: shorter than wings. Pod: 5-15 x 1-2 cm., 2-5-seeded, cylindrical or flattened, glabrous or pubescent. Seeds: 10-30 mm., flat, green. Fl. February-May.

3.1.8. Vicia narbonensis L.

Annual, subglabrous to sparingly pubescent or hirsute, 15-50 cm. Stems: ascending, procumbent to erect, branched, thick, angular. Leaves: 1.5-9 cm.; stipules: semihastate to semiorbicular, entire or dentate to incised, with a dark nectary spot beneath; tendrils: simple or somewhat branched, lacking in the lower leaves; leaflets: (1-) 2-3 (-4)-paired, 1-6x1-3 cm., subsessile, elliptical or oblong-lanceolate or ovate to obovate, obtuse or rarely acute, rounded or truncate to retuse, mucronulate, entire or dentate-serrate at margin or denticulate near apex, sparingly to densely hairy, especially along nerves, ciliate at margin. Peduncles: very short and thick. Racemes: (1-) 3-6-flowered. Flowers: 1.8-3.2 cm., short-pedicelled, deflexed to erect. Calyx: 0.7-1.3 cm., sparingly hairy; tube with oblique rim; teeth: unequal, the lower teeth longer, as long as tube, mostly ciliate. Corolla: 2-2.5 times as long as calyx, purple-violet; standard longer and keel shorter than wings. Style: hairy at apex. Pod: 3.5-6.5x0.8-1.5 cm., short-stipitate, flattened, linear to oblong-rhomboidal, curved and beaked, hairy or glabrescent, ciliate and tuberculate-denticulate at margin, often nerved. Seeds: 4-6 mm., subglobular, brown-black, more or less smooth; hilum: oblong-elliptical to oblong-ovate, whitish. Fl. February-June.

3.1.9. Lens culinaris Medik.

Annual, 16-20 cm., hairy. Stems: few to many, erect, sparingly branching, angular. Leaves: paripinnate, at least part of them terminating in a branched tendril; stipules: small, lanceolate, entire; leaflets: 3-7-paired, 0.8-1.5 x 0.4-0.6 cm., oblong-linear to linear. Peduncles: shorter than leaves, ending in an awn up to 1 cm. Racemes: 1 (-2)-flowered. Flowers: 4-6 mm. Calyx: short-campanulate; teeth: much longer than tube, nearly as long as or longer than corolla, almost equal, filiform-subulate. Corolla: white, rarely pink or violet. Staminal tube: oblique. Pod: 0.7-1.2 x 0.3-0.5 cm., deflexed, ovate-rhombic. Seeds: 1-2, lenticular, rarely almost globular. Fl. April.

3.2. Molecular Characterization of The Studied Species

Specific sites of DNA; ITS-1 spacer, 5.8S rDNA and ITS-2 spacer in four genera of Leguminosae family as well as the outgroup, *S. dominica* (labiatae), were amplifed using universal primers 18S and 28S. On an agarose gel, the PCR products obtained from genomic DNA, yielded a single band of approximately 720 bp for all of the tested species including *S. dominica* (Figure 1).

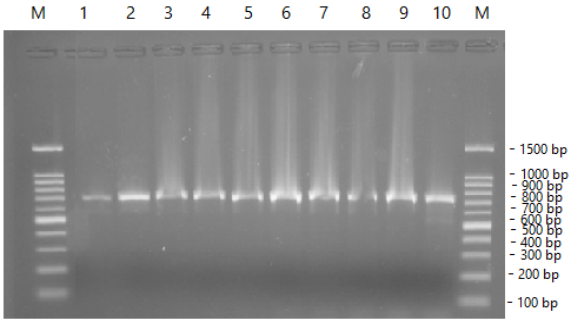

Figure 1. Agarose gel electrophoresis analysis showing the detection of amplified ITS region of different species of Leguminosae family as well as *S. dominica* as an outgroup. Lanes: M, 50 bp DNA marker; 1, *Lathyrus aphaca*; 2, *P. fulvum*; 3, *P. sativum*; 4, *V. palaestina*; 5, *V. peregrina*; 6, *V. sativa*; 7, *V. faba*; 8, *V. narbonensis*; 9; *Lens culinaris* and 10, *S. dominica*.Thirty-five classified samples of Leguminosae species belonging to *Lathyrus*, *Pisum*, *Vicia* and *Lens* genera were sequenced.

The distance matrix and phylogenetic tree of the amplified ITS region were established among the nine

Leguminosae species under investigation (Figure 2 and Table 2). The phylogenetic tree was constructed according to the similarity among the resulted sequences of the studied Leguminosae species; where the bootstrap consensus tree was inferred from 1000 replicates. The sequence information was between 663–683 bp. The obtained sequences were further registered in the GenBank database (https://www.ncbi.nlm.nih.gov) under the accession numbers (KJ864924, KJ864925 and KJ864928-KJ864961). Phylogenetic analysis in the current work revealed that the ITS sequences of Leguminosae species of interest with the highest average of intraspecies genetic divergence (9.9 %) was recorded between *V. palaestina* (KJ864941) and *P. sativum* (KJ864944) and *P. fulvum* (KJ864933, KJ864933 and KJ864933). While, the low average of intraspecies genetic divergence (1.9 %) was recorded between *V. faba* (KJ864957, KJ864958) and *V. peregrina* (KJ864954) (Table 2).

Based on the obtained phylogenetic tree, two main clades were revealed. Clade I included five species of *Vicia* genus: *V. peregrina*, *V. faba*, *V. narbonensis*, *V. palaestina* and *V. sativa*, in the same order in the phylogenetic tree respectively, as well as *Lens culinaris*. Clade II included *P. sativum*, *P. fulvum* and *Lathyrus aphaca*. Furthermore, clade I was subdivided into two subclades: a and b. Subclade Ia had five species, four of them belong to the genus *Vicia* and the fifth one was *Lens culinaris*. However, *V. sativa* occupied a basal position in the clade I. This may confirm that *V. sativa* represents a taxon distantly related to all other species of *Vicia*. *Lens culinaris* belongs to the subclade Ia. It was close to the species of *Vicia*, and appeared basally as sister to many *Vicia* species, in particular, *V. palaestina.*

The results of this research showed that all of the studied cultivated (KJ864947, KJ864948, KJ864950, and KJ864951) and the wild type (KJ864949) samples of *V. sativa* were clustered close to each other in the same group (100 % bs). Moreover, all of *Vicia* species could be considered as sisters to each other according to their botanical and molecular properties.

However, clade II was subdivided into two subclades: c and d, which were represented by the two *Pisum* studied species and *Lathyrus aphaca*, respectively (68.9 % bs). A high molecular similarity between them was observed, which was more than their resemblance to other genera. The two *Pisum sativum* and *P. fulvum* species were near to each other in the constructed phylogenetic tree in this study (100 % bs).

Finally, the current work data confirm that the species of *Pisum* genus formed a monophyletic group as all species of *Pisum* genus were clustered into the same group and had the same ancestor. On the other hand, *V. sativa* clade splits from the other closely- related species in the same genus. As a result, the genus *Vicia* was considered as a paraphyletic one. However, *S. dominica* was found to be quite divergent, and did not fall in any of the major clusters (Figure 2).

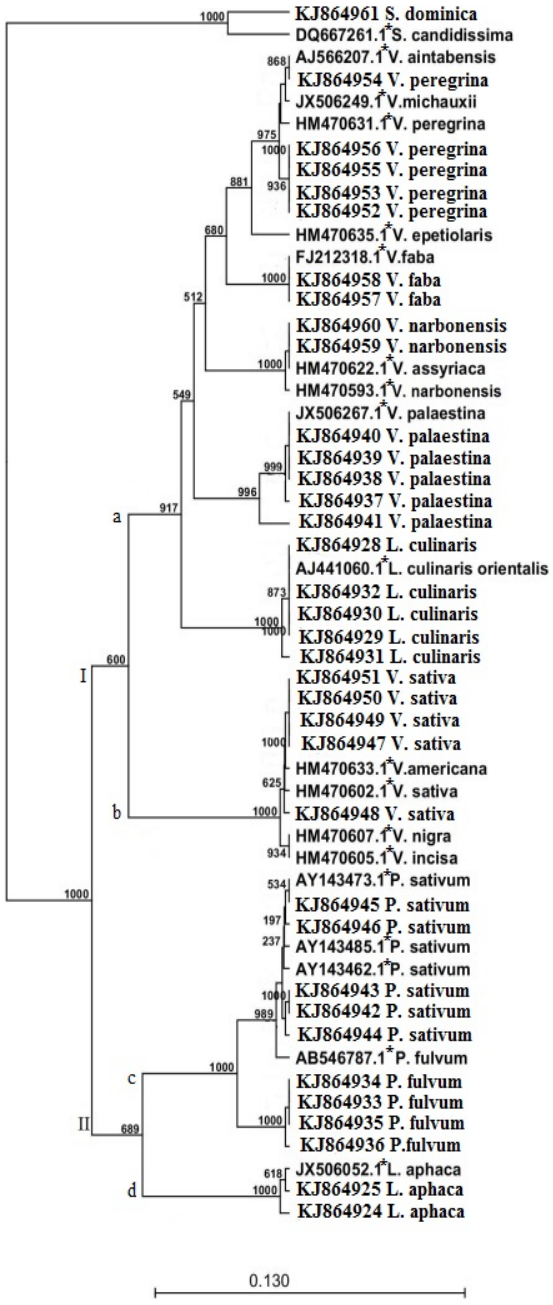

Figure 2. Phylogenetic analysis by UPGMA method based on ITS site. Sequence of some Palestinian Leguminosae species (*Lathyrus aphaca, P. fulvum, P. sativum, V. palaestina, V. peregrina, V. sativa, V. faba, V. narbonensis* and *Lens culinaris*) as well as *S. dominica* as an outgroup were used for phylogenetic analysis. Reference sequences belonging to species of Leguminosae family (denoted by asterisk) were retrieved from GenBank. The bootstrap consensus tree was inferred from 1000 replicates. The latin numbers (I, II) represent the clades, where the following letters (a, b, c and d) represent the subclades of the phylogenetic tree.

Table 2. Genetic differences between ITS region sequences derived from studied Palestinian Leguminosae species. DNA distances were created by K2P model using MEGA software version 5.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34
1. L._aphaca KJ864924																																		
2. L._aphaca KJ864925	0.005																																	
3. L. culinaris KJ864928	0.060	0.059																																
4. L._culinaris KJ864929	0.060	0.059	0.000																															
5. L. culinaris KJ864930	0.060	0.059	0.000	0.000																														
6. L. culinaris KJ864931	0.064	0.062	0.003	0.003	0.003																													
7. L. culinaris KJ864932	0.060	0.059	0.000	0.000	0.000	0.003																												
8. P. fulvum KJ864933	0.059	0.057	0.070	0.070	0.070	0.074	0.070																											
9. P._fulvum KJ864934	0.059	0.057	0.070	0.070	0.070	0.074	0.070	0.000																										
10. P. fulvum KJ864935	0.059	0.057	0.070	0.070	0.070	0.074	0.070	0.000	0.000																									
11. P. fulvum KJ864936	0.060	0.059	0.072	0.072	0.072	0.076	0.072	0.002	0.002	0.002																								
12. V. palaestina KJ864937	0.066	0.064	0.044	0.044	0.044	0.047	0.044	0.081	0.081	0.081	0.081																							
13. V. palaestina KJ864938	0.064	0.062	0.042	0.042	0.042	0.046	0.042	0.079	0.079	0.079	0.079	0.002																						
14. V. palaestina KJ864939	0.064	0.062	0.042	0.042	0.042	0.046	0.042	0.079	0.079	0.079	0.079	0.002	0.000																					
15. V. palaestina KJ864940	0.064	0.062	0.042	0.042	0.042	0.046	0.042	0.079	0.079	0.079	0.079	0.002	0.000	0.000																				
16. V. palaestina KJ864941	0.079	0.077	0.057	0.057	0.057	0.060	0.057	0.095	0.095	0.095	0.094	0.015	0.014	0.014	0.014																			
17. P. sativum KJ864942	0.062	0.061	0.070	0.070	0.070	0.074	0.070	0.026	0.026	0.026	0.028	0.079	0.078	0.078	0.078	0.093																		
18. P. sativum KJ864943	0.062	0.061	0.070	0.070	0.070	0.074	0.070	0.026	0.026	0.026	0.028	0.079	0.078	0.078	0.078	0.093	0.000																	
19. P. sativum KJ864944	0.064	0.062	0.070	0.070	0.070	0.074	0.070	0.028	0.028	0.028	0.030	0.081	0.079	0.079	0.079	0.095	0.092	0.092																
20. P. sativum KJ864945	0.061	0.059	0.068	0.068	0.068	0.072	0.068	0.024	0.024	0.024	0.026	0.078	0.076	0.076	0.076	0.091	0.092	0.092	0.093															
21. P. sativum KJ864946	0.062	0.061	0.070	0.070	0.070	0.074	0.070	0.026	0.026	0.026	0.028	0.080	0.078	0.078	0.078	0.093	0.093	0.093	0.095	0.092														
22. P. sativum KJ864947	0.060	0.059	0.046	0.046	0.046	0.049	0.046	0.070	0.070	0.070	0.072	0.049	0.048	0.048	0.048	0.062	0.074	0.074	0.076	0.072	0.074													
23. P. sativum KJ861948	0.061	0.062	0.049	0.049	0.049	0.053	0.049	0.073	0.073	0.073	0.075	0.053	0.051	0.051	0.051	0.062	0.077	0.077	0.079	0.076	0.078	0.003												
24. P. sativum KJ864949	0.060	0.059	0.046	0.046	0.046	0.049	0.046	0.070	0.070	0.070	0.072	0.049	0.048	0.048	0.048	0.062	0.074	0.074	0.076	0.072	0.074	0.000	0.003											
25. P. sativum KJ864950	0.060	0.059	0.046	0.046	0.046	0.049	0.046	0.070	0.070	0.070	0.072	0.049	0.048	0.048	0.048	0.062	0.074	0.074	0.076	0.072	0.074	0.000	0.003	0.000										
26. P. sativum KJ864951	0.060	0.059	0.046	0.046	0.046	0.049	0.046	0.070	0.070	0.070	0.072	0.049	0.048	0.048	0.048	0.062	0.074	0.074	0.076	0.072	0.074	0.000	0.003	0.000	0.000									
27. V. peregrine KJ864952	0.057	0.055	0.037	0.037	0.037	0.040	0.037	0.066	0.066	0.066	0.068	0.031	0.030	0.030	0.030	0.044	0.066	0.066	0.068	0.065	0.066	0.031	0.035	0.031	0.031	0.031	0.000							
28. V. peregrine KJ864953	0.057	0.055	0.037	0.037	0.037	0.040	0.037	0.066	0.066	0.066	0.068	0.031	0.030	0.030	0.030	0.044	0.066	0.066	0.068	0.065	0.066	0.031	0.035	0.031	0.031	0.031	0.000	0.000						
29. V. peregrine KJ864954	0.059	0.057	0.035	0.035	0.035	0.039	0.035	0.064	0.064	0.064	0.066	0.030	0.028	0.028	0.028	0.042	0.064	0.064	0.066	0.063	0.065	0.030	0.033	0.030	0.030	0.030	0.002	0.002	0.002					
30. V. peregrine KJ864955	0.057	0.055	0.037	0.037	0.037	0.040	0.037	0.066	0.066	0.066	0.068	0.031	0.030	0.030	0.030	0.044	0.066	0.066	0.068	0.065	0.066	0.031	0.035	0.031	0.031	0.031	0.000	0.000	0.000	0.002				
31. V. peregrine KJ864956	0.057	0.055	0.037	0.037	0.037	0.040	0.037	0.066	0.066	0.066	0.068	0.031	0.030	0.030	0.030	0.044	0.066	0.066	0.068	0.065	0.066	0.031	0.035	0.031	0.031	0.031	0.000	0.000	0.000	0.002	0.000			
32. V. faba KJ864957	0.064	0.062	0.040	0.040	0.040	0.044	0.040	0.070	0.070	0.070	0.072	0.042	0.040	0.040	0.040	0.055	0.070	0.070	0.072	0.068	0.070	0.033	0.037	0.033	0.033	0.033	0.021	0.021	0.021	0.019	0.021	0.021		
33. V. faba KJ864958	0.064	0.062	0.040	0.040	0.040	0.044	0.040	0.070	0.070	0.070	0.072	0.042	0.040	0.040	0.040	0.055	0.070	0.070	0.072	0.068	0.070	0.033	0.037	0.033	0.033	0.033	0.021	0.021	0.021	0.019	0.021	0.021	0.000	
34. V. narbonensis_KJ864959	0.068	0.066	0.048	0.048	0.048	0.051	0.048	0.083	0.083	0.083	0.085	0.047	0.046	0.046	0.046	0.060	0.080	0.080	0.080	0.078	0.080	0.049	0.049	0.049	0.049	0.049	0.039	0.039	0.039	0.037	0.039	0.039	0.038	0.038
35. V._narbonensis_KJ864960	0.068	0.066	0.048	0.048	0.048	0.051	0.048	0.083	0.083	0.083	0.085	0.047	0.046	0.046	0.046	0.060	0.080	0.080	0.080	0.080	0.080	0.049	0.049	0.049	0.049	0.049	0.039	0.039	0.039	0.037	0.039	0.039	0.038	0.038	0.000

4. Discussion

Evolutionary relationship illustration and reconstruction among different organisms have been among the hot topics of research lately. The phylogenetic relationship construction was based mainly on the accumulation of DNA sequence data in GeneBank targeting a new genetic classification that often conflicts with the traditional taxonomical tools. Nuclear ribosomal ITS sequence data have great potential to resolve plant phylogenies at different taxa levels. Upon that they were used to detect the phylogenetic relationship among several wild and cultivated plant species as in *Allium* species (Gurushidze *et al*., 2007) as well as among different legume species (Mayer and Bagga, 2002; Sonnante *et al*., 2003; Steele and Wojciechowski, 2003). Nevertheless, until now no similar analysis on the phylogenetic relationship for Leguminosae species has been reported in Palestine using molecular techniques such as the ITS region analysis.

The constructed phylogenetic tree in this study showed that some reference species retrieved from GenBank database (NCBI) clustered together such as *V. assyriaca* and *V. narbonensis* or *V. michauxii, V. aintabensis* and *V. peregrina* or *V. american* and *V. sativa*. This may indicate that these species, which clustered together, have a close phylogenetic relationship.

In this research, *Lens culinaris* formed a cluster within *Vicia* genus, where this species was closest to *V. palaestina* more than any other species of *Vicia* (91.7 % bs). The continuum morphological properties between *Vicia palaestina* and *Lens culinaris* is a main conflict in their discrimination from each other. Since *Vicia palaestina* and *Lens culinaris* are characterized by overlapping in their morphological characters, branched stems, branched tendrils and the number of seeds, as well as the number of leaflets, the number of raceme flowers and the size and shape of the stipules were observed. The molecular out-finding coincides with the resemblance of their morphological properties. These two species are similar to each other, except in the hairy style and the seed shape. Style is hairy all around or only on the lower side in *V. palaestina*. On the other hand, style is hairy on the upper side in *Lens culinaris*. In addition, they varied in having round and compressed seeds respectively. Therefore, in spite of this morphological discrimination, the recorded molecular data strongly supported the close relationship between them, which lead to the recommendation of their reclassification in the same genus.

The results of the current research agreed with previous studies in that *Lens* is a close genus to *Vicia*, as they formed a clade near each other based on ITS sequences (Foladi *et al*., 2013; Steele and Wojciechowski, 2003). Another study indicated that *V. faba* and *V. narbonensis* are sisters to each other, as the obtained bootstrap supports the replacement of *V. faba* into the *V. narbonensis* group (Leht, 2009). The previous study result supports those obtained in this work, as *V. peregrina, V. faba* and *V. narbonensis* were clustered with each other in the phylogenetic tree confirming their relationship to each other. On the other hand, results showed that *V. sativa* was farther similar to the other *Vicia* species due to its separation from other species of *Vicia* genus.

Furthermore, the location of *V. sativa* and *Lens culinaris* in different subclades in the resulted phylogenetic tree in this study agrees with their different morphological properties. In addition, the results pointed out that the cultivated and wild samples of *V. sativa* were clustered close to each other in the same group. This indicates the genetic closeness between wild and cultivated species, revealing the ability to consider the wild species as the origin of the cultivated ones. Nevertheless, this can be taken into account in the absence of hybridization occurrence or human intervention.

The subclade represented by *Lathyrus aphaca* is sister to the one comprised of *P. sativum* and *P. fulvum* as they formed a monophyletic group in the phylogenetic tree. This molecular analysis is in harmony with the common feature between them as both have large stipules, which are missing in *Vicia* and *Lens* genera. The obtained results are compatible with previous studies (Steele and Wojciechowski, 2003).

Two species of *Pisum* genus; *P. sativum* and *P. fulvum* formed one subclade reflecting the close relationship between them estimating that the wild *P. fulvum* is the origin of the cultivated *P. sativum* as was reported previously by (Schaefer *et al*., 2012). However, other studies showed a contradiction as *P. fulvum* diverges from other *Pisum* genus species (Palmer *et al*., 1985; Polans and Saar, 2002; Saar and Polans, 2000). Therefore, more studies considering a wider spectrum of *Pisum* species are still required to clarify this taxonomical issue. Nevertheless, the purple flowered sample of *P. sativum* was farther from the other white flowered samples. As a result, different phenotypes as different flower colors can't be considered as different species (Zohary, 1987). This complex relationship between phenotypes and genotypes confirmed by the genetic mechanisms controlling the floral number and shape is apparently unstable, resulting in a fluctuating asymmetry (Friesen *et al*., 1997). As a result, it is not easy to establish a direct relation between the phenetic variations and the genetic ones (Treu *et al*., 2001). Accordingly, further studies related to that aspect could be conducted to provide a more elaborate clear view.

The genus *Vicia* did not form a monophyletic group; instead it formed a paraphyletic one with *Lens*. This result is consistent with a previous report indicating that genus *Vicia* formed a paraphyletic group with *Lens*, *Pisum*, and *Lathyrus* (Steele and Wojciechowski, 2003) and was confirmed by obtaining a monophyletic *Vicia* by transferring *Lens* and *Lathyrus saxatilis* to *Vicia* genus (Schaefer *et al*., 2012).

Therefore, the phylogenetic relationship among plant species using ITS sequencing is an effective method for identifying unknown plant specimens that don’t have one or more essential parts such as flowers, fruits, etc., without which, the accurate identification and classification would be sometimes impossible. However, the classification of a new species according to the resulted clusters in the phylogenetic tree at family, genus and species levels is possible accurately by referring to the GenBank data via the use of small amounts of leaves.

5. Conclusion

Morphological and molecular properties of Palestinian *Lens culinaris* and *V. palaestina* revealed the close relationship between these two species. This indicates that the shape of the seed and the hairy characters of style are not enough to separate these two species into two different genera. Therefore, it is recommended to transfer *Lens culinaris* into *Vicia* genus based on the morphological and molecular characterization of this species. Accordingly, the new classification of *Lens culinaris* is proposed to be *Vicia culinaris*. However, further studies on other different species of the Leguminosae family are needed to provide more information on the relationship among the Leguminosae closely-related genera and species.

Conflict of Interest

No conflicts of interest have been declared by the authors

References

Andrea P. 2011. Identification, collection and agro-morphological characterization of lentil (*Lens culinaris* M.) landraces of Molise. Doctoral thesis, University of Molise, Italy.

Erskine W, Muehlbaue FR, Sarker A and Sharma B. 2009. **The Lentil: Botany, Production and Uses**. Wallingford, Oxford, UK.

Foladi F, Salimpour F, Sharifnia F and Ghanavati F. 2013. Phylogenetic study of tribe *Vicieae* based on internal transcribed spacer (ITS). *Ann Biol Res.*, **4**:75-97.

Friesen N, Fritsch R and Bachmann K. 1997. Hybrid origin of some ornamentals of *Allium* subgenus *Melanocrommyum* verified with GISH and RAPD. *Theor Appl Genet*., **95**:1229-1238.

Grassi F, Labra M and Sala F. 2006. **Introduzione alla Biodiversità del Mondo Vegetale**. Piccin-Nuova Libraria, Italy.

Gurushidze M, Mashayekhi S, Blattner F, Friesen N and Fritsch R. 2007. Phylogenetic relationships of wild and cultivated species of *Allium* section *Cepa* inferred by nuclear rDNA ITS sequence analysis. *Plant Syst Evol.*, **269**:259-269.

Ladizinsky G and Sakar D. 1982. Morphological and cytogenetical characterization of *Vicia montbretii* Fisch. & Mey. (Synonym: *Lens montbretii* [Fisch. & Mey.] Davis & Plitmann). *Botanic J Linnean Soc*., **85**: 209-212**.**

Leht M. 2009. Phylogenetics of *Vicia* (Fabaceae) based on morphological data. *Feddes Repert.*, **120**: 379-393.

Mayer M and Bagga S. 2002. The phylogeny of *Lens* (Leguminosae): new insight from ITS sequence analysis. *Plant Syst Evol*., **232**:145-154.

Muir G, Fleming C and Schlotterer C. 2001. Three divergent rDNA clusters predate the species divergence in *Quercuspetraea* (Matt.) Liebl. and *Quercusrobur*L. *Mol Biol Evol*., **18**:112-119.

Nickrent D and Patrick J. (1998). The nuclear ribosomal DNA intergenic spacers of wild and cultivated soybean have low variation and cryptic subrepeats. *Genome*., **41**:183-191.

Palmer J, Jorgensen R and Thompson W. 1985. Chloroplast DNA variation and evolution in *Pisum*: patterns of change and phylogenetic analysis. *Genetics*., **109**:195–213.

Penteado M, Garcia P and Perez M. 1996. Genetic variability and mating system in three species of the Genus *Centrosema*. *J Hered*., **87**:124-130.

Polanco C and Perez M. 1997. Intergenic ribosomal spacer variability in hexaploidoat cultivars and landraces. *Heredity*., **78**:115-123.

Polanco C and Perez M. 1995. Length polymorphism in the ribosomal DNA intergenic spacer of rye and slender wild oats. *Heredity*., **86**:402-407.

Polans N and Saar D. 2002. ITS sequence variation in wild species and cultivars of pea. *Pisum Genet*., **34**:9-13.

Saar D and Polans N. 2000. ITS sequence variation in selected taxa of *Pisum*. *Pisum Genet*., **32**:42-45.

Schaefer H, Hechenleitner P, Santos-Guerra A, Menezesde Sequeria M, Pennington R, Kenicer G and Carine M. 2012. Systematics, biogeography, and character evolution of the legume tribe Fabeae with special focus on the middle-Atlantic island lineages. *BMC Evol Bio*l., **12**:250.

Sonnante G, Galasso I and Pignone D. 2003. ITS sequence analysis and phylogenetic inference in the genus *Lens* Mill. *Ann Bot*., **91**:49-54.

Steele K P and Wojciechowski MF. 2003. Phylogenetic analyses of tribes *Trifolieae* and *Vicieae*, based on sequences of the plastid gene *matK* (Papilionoideae**:** Leguminosae). In: Klitgaard B and Bruneau A (Eds.), **Advances in Legume Systematics**. Part10. Royal Botanic Gardens, Kew Bull, pp.355-370.

Treu R, Holmes D, Smith B, Astley D, Johson M and Trueman L. 2001. *Allium ampeloprasum* var. *babingtonii* (Alliaceae): an isoclonal plant found across a range of habitats in S.W. England. *Plant Ecol*., **155**:229-235.

Zhang Q, Saghai M and Allard R. 1990. Effects of adaptedness of variations in ribosomal DNA copy number in populations of wild barley (*Hordeum Vulgare* ssp. *Spontaneum*). *Proc Natl Acad Sci U S A.*, **87**: 8741-8745.

Zohary M. 1987. **Papilionaceae**. Flora Palaestina. The Israel Academy of Science and Humanities, Jerusalem, Palestine. 34-223.

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Phylogenetic Relationships within the Tribe Hippotragini (Antilopinae: Bovidae) Based on Mitochondrial Genome

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Abstract

In this survey, phylogenetic relationships within the tribe Hippotragini were re-evaluated using 132 gene sequences and ten complete mitogenomes. Based on the complete mitochondrial genomes, the average base composition of mtDNA sequences was 26.9 % T, 26.3 % C, 33.4 % A, and 13.4 % G, showing a strong AT bias (60.3 %). The phylogenetic trees were constructed using maximum likelihood and Bayesian inference methods, and were found to have very identical topologies. The results of this study revealed that this tribe is a strongly supported monophyletic group, and in all of the phylogenetic trees, except for COXI, *Addax nasomaculatus* is considered as a sister group for the monophyletic group of the genus *Oryx*. All of the species belonging to the genus *Hippotragus* constructed another monophyletic clade. Besides, within the monophyletic group of the genus *Oryx*, there are probably two distinct evolutionary pathways, i.e. *O. gazella* was located as a sister taxon of other species. Also, based on the whole mitogenomes, the extinct blaubok (*H. leucophaeus*), the endangered giant sable (*H. niger variani*), and the southern sable (*H. niger niger*), showed very close phylogenetic relationships and the roan antelope (*H. equinus*) is nested as their sister taxon.

KeyWords: Hippotragini, Phylogeny, Re-evaluation, mtDNA, Bovidae, Antilopinae.

1. Introduction

Taxonomically, the tribe Hippotragini belongs to the Family Bovidae, Subfamily Antilopinae (Wilson and Reeder, 2005; Castelló, 2016). Historically, these antelopes were considered to be a separate subfamily within the family Bovidae, but current molecular evidence suggests that they are best relegated to a tribal level within the subfamily Antilopinae (one of two subfamilies within the Family Bovidae) (Holland, 2015; Mathee and Davis, 2001; Kuznetsova *et al*., 2002; Hassanin and Douzery, 2003; Ropiquet, 2006; Hassanin *et al*., 2012).

These "horse-like" antelopes consist primarily of large grazing antelopes with large horns: oryxes, addax, sable, and roan antelopes. Hippotragini species are restricted to Africa and the Arabian Peninsula, and are primarily grazers. Most species live in arid habitats and have an erect mane along the nape of the neck (Castelló, 2016; Holland, 2015). The females are almost or quite as well-horned as the males. The horns, which are ringed, are generally more or less parallel and the frontal sinuses extend into the horn pedicels (Groves and Grubb, 2011).

As antelopes specialized in harsh arid conditions, all species have very high-crowned hypsodont teeth and a bending braincase to maximize the grazing efficiency (Prothero and Foss, 2007). Also, they employ a highly efficient thermoregulation mechanism ("nasal sweating") to effectively cool their bodies and prevent water loss (Kingdon and Hoffman, 2013; Holland, 2015). All species are gregarious and form mostly small herds (Holland, 2015).

The tribe Hippotragini consists of three genera: *Addax*, *Oryx*, and *Hippotragus.* The genus *Hippotragus* consists of three species: *H. leucophaeus* (blaubok) (extint), *H. equinus* (roan antelope), and *H. niger* (sable) (Groves and Grubb, 2011). Four subspecies are usually recognized: *H. niger niger*, *H. niger kirkii*, *H. niger anselli*, *H. niger roosevelti*, and the isolated Giant Sable (*H. niger variani*) from Angola (Pitra *et al*. 2002). In some references, *H. niger roosevelti* and *H. niger variani* have been elevated to full species (Groves and Grubb, 2011). The genus *Addax* consists of a single species: *A. nasomaculatus* (Groves and Grubb, 2011). The genus *Oryx* consists of six species: *O. beisa* (Beisa oryx), *O. callotis* (fringe-eared oryx), *O. gazelle* (gemsbok), *O. dammah* (scimitar- horned oryx), *O. leucoryx* (Arabian oryx), and *O. gallarum* (Groves and Grubb, 2011).

The first ancestors of the Hippotragini, Alcephalini, and Caprini tribes evolved in Eurasia during the early Miocene (Turner and Anton, 2004; Prothero and Foss, 2007; Kingdon and Hoffman, 2013; Geraads *et al*., 2008; Holland, 2015). Fossil genera including *Palaeoryx*, *Tragoreas*, and *Damalavus*, were intermediates between Hippotragini, Alcephalini, and Caprini (Prothero and Foss, 2007; Holland, 2015). Late Miocene crown members of Hippotragini are found in Northern Africa and include *Tchadotragus* and *Saheloryx* (Geraads *et al.*, 2008). It was not until the Pliocene that the Hippotragini diversity exploded in Eurasia as a response to the cooling

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temperatures and the advancement of grasslands (Holland, 2015).

There has been a considerable advancement in sequencing mammalian mtDNA molecules and their analysis. Mitochondrial DNA (mtDNA) possesses several favourable characteristics, including large quantity in the cell simplicity of extraction, small genome size, haploid, maternal inheritance with extremely low probability of paternal leakage, higher mutation rate than nuclear DNA, and the amenability to change mainly through mutation rather than recombination. All these features make mtDNA a useful and one of the most frequently used markers for evolutionary and phylogenetic studies (Ghassemi-Khademi, 2017).

Several researchers (Amir and Shobrak, 2011; Hassanin *et al*., 2012; Matthee and Davis, 2001; Elmeer *et al*., 2012; Iyengar *et al*., 2006; Khan *et al*., 2011; Kuznetsova *et al*., 2002; Masembe *et al*., 2006; Matthee and Robinson, 1999; Osmers *et al*., 2012; Khan *et al*., 2008; Arif *et al*., 2009; Themudo *et al*., 2015; Yang *et al*., 2013) have studied the phylogenetic relationships of species belonging to the tribe Hippotragini using different genes, but there is no comprehensive re-evaluation study of the phylogenetic relationships within the tribe of Hippotragini using different mitochondrial genes. Determining the phylogenetic relationships between the genera belonging to this subfamily can be an effective step in planning for the conservation of these animals in the wild. Most species belonging to this subfamily are in serious danger of extinction and their populations are clearly declining (Holland, 2015).

2. Materials and Methods

Gene sequences including cytochrome b (n=54), 12S ribosomal RNA (or 12S rRNA) (n=37), 16S ribosomal RNA (or 16S rRNA) (n=29), and cytochrome c oxidase I (n=12), and complete mitochondrial genome sequences (n=10) (a total of 142 sequences) belonging to the tribe Hippotragini were downloaded from NCBI (Tables 1, 2, 3, 4 and 5). The titles of the received sequences were edited by ExcaliBAR (Aliabadian *et al*., 2014); and BioEdit 7.0.5.3 software (Hall 1999) was used to create a DNA sequence alignment using Clustal W algorithm (Thompson *et al*., 1994) in all of the received sequences. In all of the studied genes, the corresponding gene sequences of *Pelea capreolus* were used as an outgroup in the analyses.

The most appropriate sequence evolution model for the given data was determined with Model test (Posada and Crandall, 1998) as implemented in the MEGA6 software (Tamura *et al*., 2013). The model with the lowest BIC (Bayesian Information Criterion) scores is considered to best describe the substitution pattern. The evolutionary history was inferred using the Maximum Likelihood method "ML" for each of the studied genes separately. The trees were calculated with the highest log likelihood. In all of the phylogenetic trees, the percentage of replicate trees, in which the associated taxa are clustered together in the bootstrap test (1000 replicates), were shown next to the branches (Felsenstein 1985). In all of the above-mentioned analyses, all positions containing gaps and missing data were eliminated, and were conducted in MEGA6 (Tamura *et al*., 2013). Also, the robustness of clades was calculated by the bootstrap method. In this study, 50-60 % was considered as a weak support (as bootstrap values), 61-75 % as a moderate support, 76-88 % as a good support, and ≥89 % as strong support values (Retrieved from: Win *et al*., 2017; with minor modification).

Table 1. The accession numbers of cytochrome b genes and scientific names of studied species from the tribe Hippotragini (n=54) received from GenBank (www.ncbi.nlm.nih.gov)(n=55).

Scientific Names	Accession Numbers	Scientific Names	Accession Numbers
Hippotragus equinus	AF022060	*Oryx leucoryx*	FJ937683
	HQ641316		JN632679
	JF728769		NC_020732
	JN632647		JN869312
	NC_020712		KC282640
Addax nasomaculatus	AF034722	*Oryx gazella*	NC_016422
	JN632591		JN632678
	KM582122		JF728779
	NC_020674	*Oryx dammah*	JF728778
	DQ138192		JN632677
Oryx beisa	DQ138193	*Pelea capreolus*	AF022055
	DQ138194	*Oryx leucoryx*	FJ937660
	DQ138195		FJ937661
	DQ138196		FJ937664
	DQ138197		FJ937665
	DQ138198		FJ937670
	DQ138199		FJ937676
	DQ138200		FJ937679
	DQ138201		FJ937680
	DQ138202		FJ937681
	DQ138203		FJ937682
	DQ138204		
	DQ138205		
	DQ138206		
	DQ138207		
	DQ138208		
	DQ138209		
	DQ138210		
	HM209249		
	JN632676		
	NC_020793		

In addition, the Bayesian analyses of the studied gene sequences were run with the parallel version of MrBayes 3.1.2 (Ronquist and Huelsenbeck, 2003) on a cluster with one processor assigned to each Markov chain under the most generalizing model (GTR+G+I) because over parametrization apparently does not negatively affect Bayesian analyses (Huelsenbeck and Ranala, 2004). Each Bayesian analysis comprised two simultaneous runs of four Metropolis-coupled Markov-chains at the default temperature (0.2). Analyses were terminated after the chains converged significantly, as indicated by the average standard deviation of split frequencies<0.01. The Bayesian inference of phylogeny was conducted for 8,000,000 generations. Seven hundred bootstrap replicates were used as ML branch support values. The posterior probabilities equal/higher than 0.95 and bootstrap supports equal/higher than 70 % were considered as strong support values (Ghassemi-Khademi, 2018). The obtained phylogenetic trees were visualized and edited by FigTree software v1.4.2 (http://tree.bio.ed.ac.uk/software/figtree/). Since all of the species belonging to a single genus were considered as a separate group, three groups were determined and the pair fixation indices (F_{ST}) among these groups was calculated based on mitogenomes using Arlequin 3.5 (Excoffier *et al*., 2005). Referring to the criterion for genetic differentiation by Wright (1978), genetic differentiation was defined as low for F_{ST}<0.05, moderate

for $0.05<F_{ST}<0.15$, high for $0.15<F_{ST}<0.25$, and very high for $F_{ST}>0.25$.

Table 2: The accession numbers of 12S ribosomal RNA (or 12S rRNA) genes and scientific names of studied species from the tribe Hippotragini (n=37) received from GenBank (www.ncbi.nlm.nih.gov)(n=38).

Scientific Names	Accession Numbers	Scientific Names	Accession Numbers
Hippotragus niger	AY670653	*Oryx dammah*	U86970
	KM245339		JN632677
	JN632648	*Oryx beisa*	JN632676
	NC_020713		NC_020793
	U86974	*Pelea capreolus*	U86994
	AF091709	*Oryx gazella*	M86500
Hippotragus leucophaeus	NC_035309		JN869312
	MF043256		NC_016422
Addax nasomaculatus	U86973		JN632678
	JN632591		KC282640
	NC_020674		FJ914296
Oryx leucoryx	FJ914314		FJ914294
	FJ914312		FJ914293
	FJ914311	*Oryx leucoryx*	FJ914292
	FJ914308		FJ914291
	FJ914306		U86971
	FJ914305		JN632679
	FJ914302		NC_020732
	FJ914299		
	FJ914297		

Table 3. The accession numbers of 16S ribosomal RNA (or 16S rRNA) genes and scientific names of studied species from the tribe Hippotragini (n=29) received from GenBank (www.ncbi.nlm.nih.gov)(n=30).

Scientific Names	Accession Numbers	Scientific Names	Accession Numbers
Addax nasomaculatus	U87023	*Oryx leucoryx*	FJ914269
	JN632591		FJ914268
	NC_020674		FJ914267
	AY122049		U87021
Hippotragus niger	U87024		JN632679
	KM245339		NC_020732
	JN632648		M86500
Hippotragus leucophaeus	NC_020713		JN869312
	NC_035309	*Oryx gazella*	NC_016422
	MF043256		JN632678
Hippotragus equinus	U87025		KC282640
	JN632647	*Oryx dammah*	U87020
	NC_020712		JN632677
Pelea capreolus	U87044	*Oryx beisa*	JN632676
			NC_020793

Table 4. The accession numbers of cytochrome c oxidase I (COX1) genes and scientific names of studied species from the tribe Hippotragini (n=12) received from GenBank (www.ncbi.nlm.nih.gov)(n=13).

Scientific Names	Accession Numbers	Scientific Names	Accession Numbers
Oryx dammah	JF444371	*Addax nasomaculatus*	JN632591
Hippotragus equinus	HQ603146		NC_020674
	HQ603145	*Hippotragus niger*	JN632648
	HQ603144		NC_020713
Oryx gazella	JF444372	*Oryx beisa*	JN632676
	JX436995	*Pelea capreolus*	JN632684
Hippotragus leucophaeus	MF043256		

Table 5. Nucleotid composition of mtDNA of studied species from the tribe Hippotragini (n=10) received from GenBank (www.ncbi.nlm.nih.gov).

Scientific name	**Accession Number**	**T**	**C**	**A**	**G**	**Total**	**References**
Addax nasomaculatus	JN632591	27.0	26.1	33.6	13.3	16751.0	(Hassanin *et al*. 2012)
Oryx gazella	JN869312	26.9	26.1	33.4	13.5	16661.0	Direct Submission
Oryx beisa	JN632676	27.0	26.1	33.5	13.3	16518.0	(Hassanin *et al*. 2012)
Oryx dammah	JN632677	27.1	26.1	33.4	13.5	16688.0	(Hassanin *et al*. 2012)
Oryx leucoryx	JN632679	27.2	26.0	33.5	13.3	16680.0	(Hassanin *et al*. 2012)
Oryx gazella	JN632678	27.1	26.0	33.5	13.4	16667.0	(Hassanin *et al*. 2012)
Hippotragus equinus	JN632647	26.5	26.6	33.6	13.3	16436.0	(Hassanin *et al*. 2012)
Hippotragus leucophaeus	MF043256	26.5	26.6	33.4	13.5	16468.0	(Themudo and Campos 2017)
Hippotragus niger	JN632648	26.6	26.5	33.3	13.6	16506.0	(Hassanin *et al*. 2012)
Hippotragus niger	KM245339	26.8	26.6	33.1	13.4	16160.0	(Themudo *et al*. 2015)
Avg.		26.9	26.3	33.4	13.4	16553.5	**********
Pelea capreolus	JN632684	*	*	*	*	*	(Hassanin *et al*. 2012)

3. Results

As the results indicated, outgroups were separated from the tribe members in all of the phylogenetic trees (Figures 1-10), implying the presence of relatively close genetic distances among tribe members. Also, in most of the phylogenetic trees, all species belonging to a single genus were clustered together. In addition, in order to show the accuracy of the phylogenetic analyses, different genera of this tribe were demarcated precisely. The average length of mitochondrial genome was calculated 16553.5 bp. In 16553.5, the average base composition of mtDNA sequences was 26.9 % T, 26.3 % C, 33.4 % A, and 13.4 % G, showing a strong AT bias (60.3 %).

In addition, in all of the phylogenetic trees, the BI posterior probability values of the tribe members were equal to 100; thus, it can be inferred that the tribe Hippotragini is a monophyletic group with the highest BI posterior probability value. Also, based on lowest BIC,

Tamura 3-parameter model (Tamura, 1992) was chosen as the best model for ML trees of 16S rRNA, COX1, and 12S rRNA genes. Hasegawa-Kishino-Yano (Hasegawa *et al*., 1985) and Kimura 2-parameter (Kimura, 1980) were the best models for complete mitochondrial genomes and cytochrome b genes, respectively.

Based on the topology of the ML phylogenetic tree of 12S rRNA sequences, the relationship of different genera belonging to the tribe Hippotragini is as follows: [{(*O. leucoryx* + (*O. dammah*) + (*O. gazella*) + (*O. beisa*) + *A. nasomaculatus*)} + {(*H. leucophaeus*) + (*H. niger*)}].

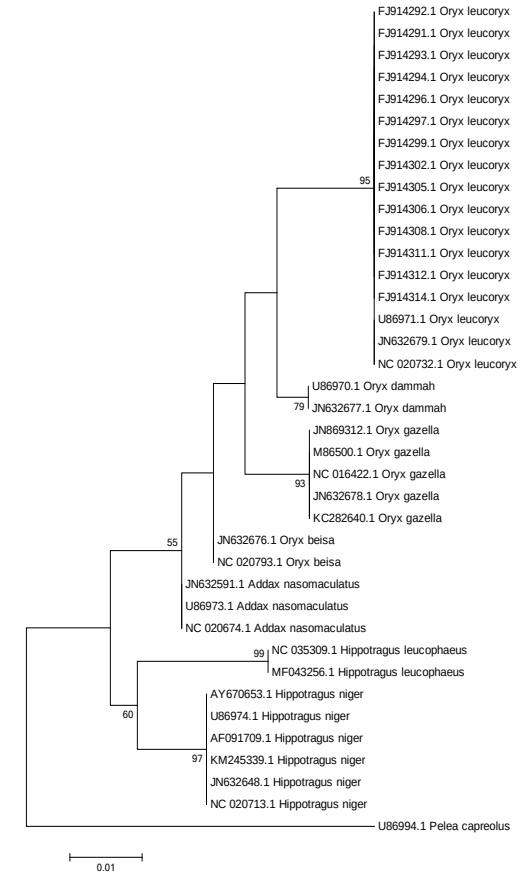

Figure 1. Maximum Likelihood tree based on Tamura 3-parameter distance using 12S ribosomal RNA (12S rRNA) sequences; the numbers on each branch correspond to bootstrap support values (percentages lower than 50 are not shown). The tree was rooted with a single *Pelea capreolus* sequences.

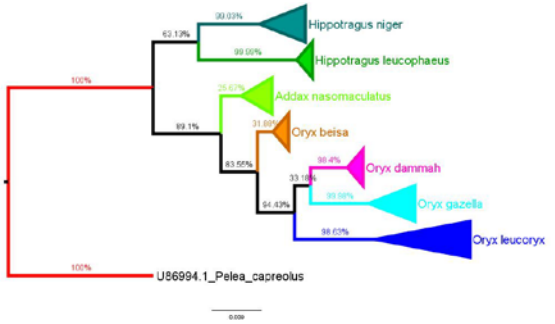

Figure 2. Bayesian phylogeny reconstructed based on 12S ribosomal RNA (12S rRNA) sequences. The values beside the branches are BI posterior probability values. The tree was rooted with a single *Pelea capreolus* sequence.

In this tree, two distinct major clusters can be distinguished. The results showed that all species belonging to the genera *Addax* and *Oryx*, have the weak (but acceptable) supported ML bootstrap (=55) and high BI posterior probability (=89.1) values. In another cluster, two species including *H. niger* and *H. leucophaeus* constructed a monophyletic group with moderate BI posterior probability (=63.13) and weak (but acceptable) supported ML bootstrap (=60) values.

Moreover, based on the topology of ML phylogenetic tree of 16S ribosomal RNA (16S rRNA) sequences, the relationship of different genera belonging to the tribe Hippotragini is as follows:

[{(*O. leucoryx* + *O.dammah*) + (*O. beisa* + *O. gazelle*) + *A. nasomaculatus*} + {(*H. niger*) + (*H. leucophaeus* + *H. equinus*)}].

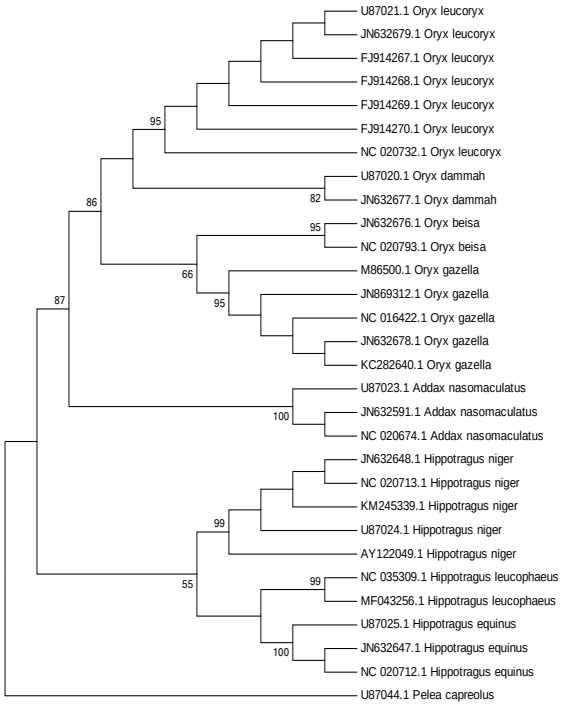

Figure 3. Maximum Likelihood tree based on Tamura 3-parameter distance using 16S ribosomal RNA (16S rRNA) sequences; the numbers on each branch correspond to bootstrap support values. (percentages lower than 50 are not shown). The tree was rooted with a single *Pelea capreolus* sequence.

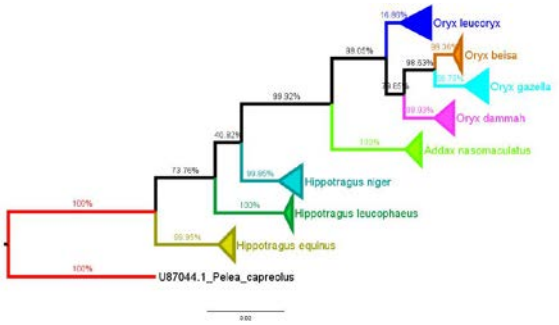

Figure 4. Bayesian phylogeny reconstructed based onbased on 16S ribosomal RNA (16S rRNA) sequences. The values beside the branches are BI posterior probability values. The tree was rooted with a single *Pelea capreolus* sequence.

In this phylogenetic tree also, two distinct major clusters can be distinguished. Where all of the sequences belonging to the genera *Oryx* and *Addax* showed good supported ML bootstrap (=87) and very high BI posterior probability (=99.92) values. In another cluster, three species including *H. niger*, *H. equines*, and *H. leucophaeus* built a monophyletic group with weak (but acceptable) supported ML bootstrap (=55). Of course in this case, the topology of the Bayesian phylogenetic tree was slightly different from the topology of ML phylogenetic tree; because in this tree, three species belonging to the genus *Hippotragus* did not construct a single monophyletic group.

Furthermore, based on the topology of ML phylogenetic tree of Cytochrome b sequences, the relationship of different genera belonging to the tribe Hippotragini is as follows:

[{((*O. beisa*)+(O.*gazella*+*O. leucoryx*)+*O.dammah*)+*A. nasomaculatus*}+{*H. equinus*}].

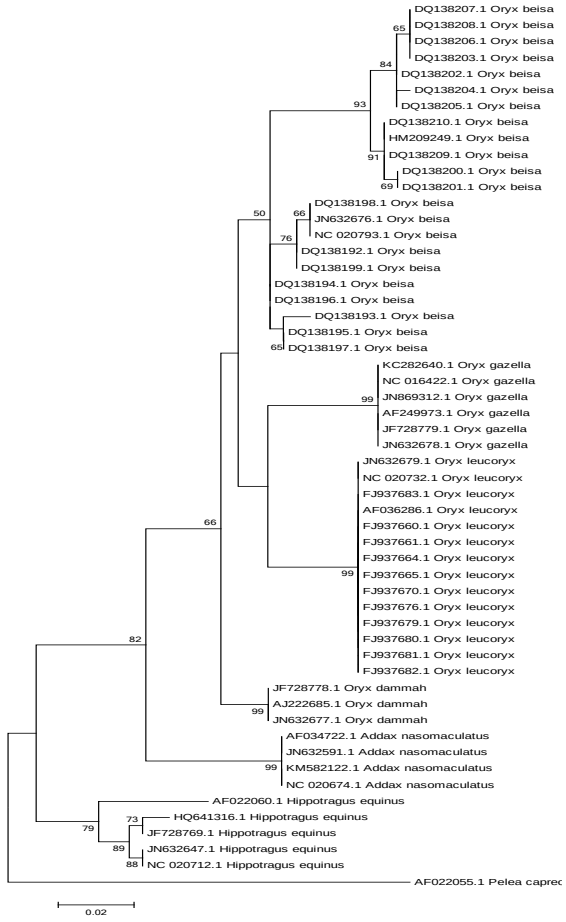

Figure 5. Maximum Likelihood tree based on Kimura 2-parameter distance using Cytochrome b sequences; the numbers on each branch correspond to bootstrap support values. (percentages lower than 50 are not shown). The tree was rooted with a single *Pelea capreolus* sequence.

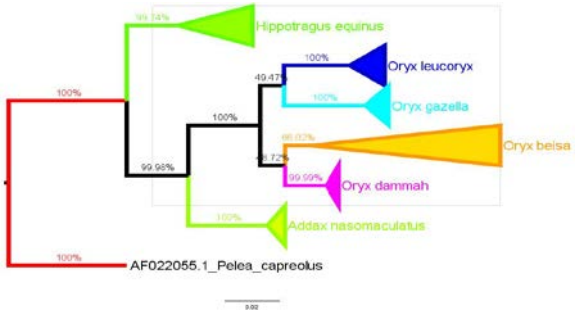

Figure 6. Bayesian phylogeny reconstructed based on Cytochrome b sequences. The values beside the branches are BI posterior probability values. The tree was rooted with a single *Pelea capreolus* sequence.

In this phylogenetic tree, there are, also, two distinct major clusters. All of the species belonging to the genera *Oryx* and *Addax* constructed a single monophyletic group with good supported ML bootstrap (=82) and very high BI posterior probability (=99.98) values. In another cluster, all of the sequences belonging to the species *H. equinus* constructed a single cluster with good supported ML bootstrap (=79) and very high BI posterior probability (=99.74) values.

Moreover, based on the topology of ML phylogenetic tree of Cytochrome c oxidase I (COXI) sequences, the relationship of different genera belonging to the tribe Hippotragini is as follows:

[{((*O. dammah* + *O. beisa*) + *A. nasomaculatus*) + *O. gazella*} +{(*H. equinus*) +((*H. leucophaeus*)+(*H. niger*))}].

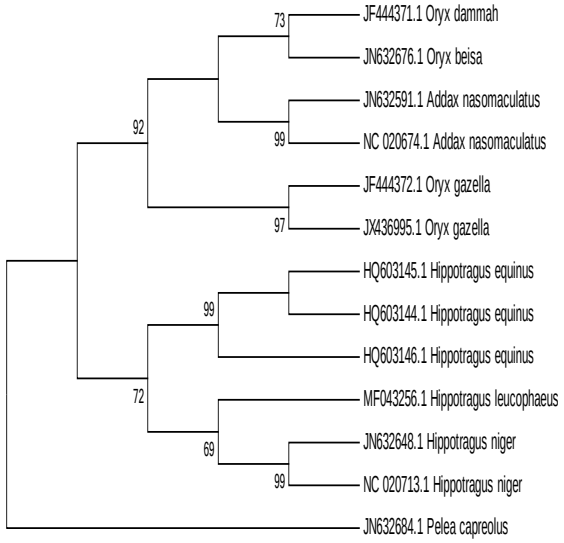

Figure 7: Maximum Likelihood tree based on Tamura 3-parameter distance using Cytochrome c oxidase I (COX1) sequences; the numbers on each branch correspond to bootstrap support values. (Percentages lower than 50 are not shown). The tree was rooted with a single *Pelea capreolus* sequence.

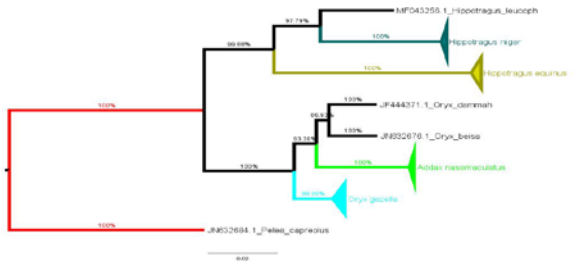

Figure 8: Bayesian phylogeny reconstructed based on Cytochrome c oxidase I (COX1) sequences. The values beside the branches are BI posterior probability values. The tree was rooted with a single *Pelea capreolus* sequence.

In this tree, there are two distinct major clusters too. All of the species belonging to the genera *Oryx* and *Addax* constructed a single monophyletic group with strong supported ML bootstrap (=92) and highest BI posterior probability (=100) values. In another major cluster, three species belonging to the genus *Hippotragus* constructed a single monophyletic cluster with moderate supported ML bootstrap (=72) and very high BI posterior probability (=99.88) values.

Eventually, based on the topology of ML phylogenetic tree of complete mitochondrial genome sequences, the relationship of different genera belonging to the tribe Hippotragini is as follows:

[{((*O. dammah* + *O. leucoryx*) + *O. beisa*) + (*O. gazella*) + *A. nasomaculatus*}+{*H. equinus* + (*H. leucophaeus* + (*H. niger*))}].

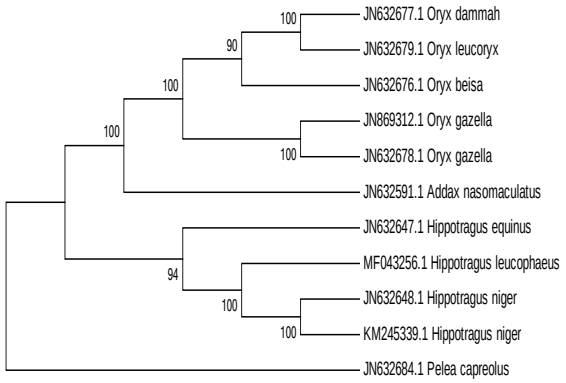

Figure 9. Maximum Likelihood tree based on Hasegawa-Kishino-Yano distance using complete mitochondrial genome sequences. The numbers on each branch correspond to bootstrap support values. The tree was rooted with a single *Pelea capreolus* sequence.

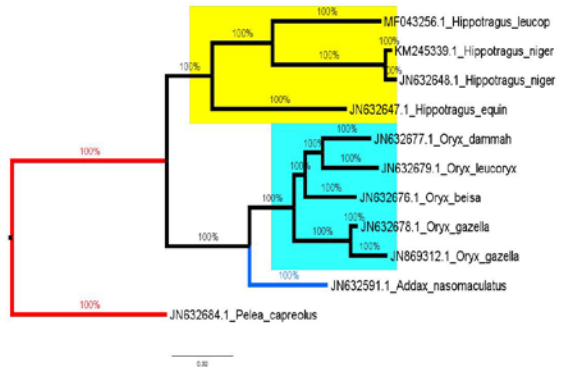

Figure 10. Bayesian phylogeny reconstructed based on complete mitochondrial genome sequences. The values beside the branches are BI posterior probability values. The tree was rooted with a single *Pelea capreolus* sequence.

Based on the topology of this tree that is probably close to reality, and similar to previous phylogenetic trees, there are two completely distinct major clusters. All of the species belonging to the genera *Oryx* and *Addax* constructed a single monophyletic group with strongest supported ML bootstrap (=100) and highest BI posterior probability (=100) values. In another major cluster, three species belonging to the genus *Hippotragus* constructed a single monophyletic cluster with strong supported ML bootstrap (=94) and highest BI posterior probability (=100) values.

Using complete mitochondrial genomes, all of the species belonging to a single genus were considered as a separate group. In addition to the outgroup, four groups were determined and phylogenetic distances between these groups were calculated. As expected, the outgroup was at a distance far from the tribe members and the ingroup. The shortest distance was obtained between the genera *Addax* and *Oryx* (=841), and the longest distance was found between the genera *Oryx* and *Hippotragus* (=1529). Also, the genetic distance between the genera *Addax* and *Hippotragus* was equal to 1486. Furthermore, genetic differentiation between populations using pairwise F_{ST} values were very high (F_{ST}>0.25). According to the results of genetic distances, the highest F_{ST} was detected between *Oryx* and *Hippotragus* (=0.484), and lowest F_{ST} was detected between the genera *Addax* and *Oryx* (0.326). The genetic differentiation between the genera *Oryx* and *Hippotragus* was significant (F_{ST} P-value=0.027) (Table 6).

Table 6. Pairwise F_{ST} based on complete mitochondrial genomes between different genera belonging the tribe Hippotragini.

Name	*Oryx*	*Addax*	*Hippotragus*
Oryx	0.00		
Addax	0.326	0.00	
Hippotragus	0.484	0.354	0.00

4. Discussion

In this study, four gene fragments in addition to complete mitochondrial genomes were used to evaluate the phylogenetic relationships within the tribe of Hippotragini. Interrelationships among Hippotragini have been largely overlooked (Holland, 2015).

In a comprehensive phylogenetic study using supertree methodology consisting of 201 source trees, Price *et al.* (2005) introduced *Hippotragus* as a sister group to *Addax*/*Oryx* clade. The results of the present study are exactly consistent with these results. In all of the phylogenetic trees, *Hippotragus* was located as a sister group to *Addax*/*Oryx* monophyletic cluster.

In another comprehensive and interesting phylogenetic research, Hassanin *et al*. (2012) evaluated the pattern and timing of diversification of Cetartiodactyla, using complete mitochondrial genomes (Hassanin *et al*., 2012). They used seven complete mtDNA sequences belonging to *H. equinus*, *H. niger*, *A. nasomaculatus*, *O. beisa*, *O. gazella*, *O. leucoryx*, and *O. dammah* for the tribe Hippotragini. The phylogenetic relationships of different species of Hippotragini were as follows:

[{*H. equinus* + *H. niger*} + {*A. nasomaculatus* + ((*O. beisa*) + (*O. gazella*) + (*O. leucoryx* + *O. dammah*))}]; and the bootstrap percentages of maximum likelihood and Bayesian trees supported a monophyletic cluster for the tribe Hippotragini (100 %) with the genus *Hippotragus* (82 %) as a sister group to *Addax*/*Oryx* (100 %) (Hassanin *et al*., 2012). Hassanin *et al*., (2012) used seven complete sequences belonging to the tribe Hippotragini, but in the present study, we added three other sequences (received from NCBI) to this seven mentioned sequences. For this reason, the phylogenetic relationships within the tribe Hippotragini in the tree in this study is slightly different than those based on seven complete mitochondrial genomes (specially in the case of the species belonging to the genus *Hippotragus*). The obtained topologies from complete mitochondrial genome provide a higher level of support than those based on individual or partial mitochondrial genes (Krzywinski *et al*., 2006) and probably the topology presented in this paper, is likely to be closer to reality.

In all of the obtained phylogenetic trees (except for cytochrome oxidase I), the genus *Addax* was a sister group of all species belonging to the genus *Oryx*. Interestingly, in addition to the complete mitochondrial genomes, this theory is also proven using other gene fragments including: 12S ribosomal RNA, 16S ribosomal RNA and Cytochrome b. Accordingly, these two genera are sister groups. This theory has also been confirmed using control region (D-loop) gene fragments (Khan *et al*., 2008b).

In another study, Yang *et al*. (2013) analyzed phylogenetic relationships within the family Bovidae based on complete mitochondrial genomes. The relationship of the species belonging to the tribe Hippotragini was as follows:

[{*H. equinus* + *H. niger*}+ {(*O. gazelle* + (*O. beisa* + O. *leucoryx*)) + (*O. dammah* + *A. nasomaculatus*)}].

In the mentioned tree, *A. nasomaculatus* was clustered with *O. dammah* and this cluster was a sister group of other species belonging to the genus *Oryx*. Moreover, Themudo *et al*., (2015) presented a similar topology; as a result, the genus *Oryx* would be considered as polyphyletic. However, all of the studied genes (except COXI) in the present study constructed a single clade for this genus, so all species belonging to the genus *Oryx* may be form a single monophyletic group, and *A. nasomaculatus* is considered as its sister group. Yang *et al*. (2013) considered Hippotragines as a subfamily (Hippotraginae), but current molecular evidences suggest that they are best relegated to a tribal level within the subfamily Antilopinae (Groves and Grubb, 2011).

Among the different species of the genus *Oryx*, the scimitar-horned oryx (*O. dammah*) and the Arabian oryx (*O. leucoryx*) showed very close phylogenetic relationships. These two species constructed a single monophyletic cluster as shown by the maximum likelihood (ML) trees of 12S rRNA and 16S rRNA, especially by the maximum likelihood and Bayesian phylogenetic trees of complete mitochondrial genomes (Ghassemi-Khademi 2017). Other studies have shown similar results (Khan *et al*., 2008c; Arif *et al*., 2009; Hassanin *et al*., 2012). These two species are probably sister species, although cytochrome b and Cytochrome c oxidase I (COX I) failed to cluster them in the ML trees.

Except for complete mitochondrial genomes, Bayesian phylogenetic trees did not cluster the scimitar-horned oryx and Arabian oryx; therefore, the maximum likelihood methods may be more superior to the Bayesian method in inferring phylogenetic relationships using gene fragments in this tribe. Likelihood-based approaches have proven to be especially powerful for inferring phylogenetic trees, but they tend to be prohibitively slow due to the requirement of multidimensional space for possible outcomes (optimal trees), in addition to the computational complexity of bootstrap repetitions (Khan *et al*., 2008c).

The genus *Oryx* is a monophyletic taxon. In the study of Hassanin *et al*. (2012), all of the species belonging to this genus have constructed a single cluster with strong bootstrap percentages of maximum likelihood (=94), but in this tree, two different clades can be distinguished where three species of *O. gazella*, *O. leucoryx*, and *O. dammah* constructed a monophyletic group, but the node of this clade has not a strong bootstrap support value (=56) and *O. beisa* is the sister taxon. In the present study, the topology of phylogenetic relationships within *Oryx* genus was different from that in Hassanin *et al*. (2012).

Based on the results of present study, Bayesian phylogenetic trees based on complete mitochondrial genomes, in addition to cytochrome b, 16S rRNA, and 12S rRNA genes, reconstructed a monophyletic group of the genus *Oryx* with high BI posterior probability values of 100, 100, 98.05, 83.55, respectively. Also, maximum likelihood phylogenetic trees based on complete mitochondrial genomes, in addition to cytochrome b and 16S rRNA genes reconstructed a monophyletic group with strong (100), moderate (66) and good (86) bootstrap support values, respectively. The monophylethic clade of *Oryx* in cytochrome b gene was not supported by acceptable bootstrap support values (percentage was lower than 50).

Based on complete mitochondrial genomes, two distinct clades have been distinguished within the genus *Oryx*, where three species including: *O. dammah*, *O. leucoryx*, and *O. beisa* form a monophyletic group with a strong support bootstrap value (=90), and two sequences belonging to *O. gazella* were located as a sister group for this monophyletic group with the highest support value (=100). This result was not illustrated in the works of Hassanin *et al*. (2012) and Yang *et al*. (2013). This result was also confirmed using cytochrome c oxidase I (COXI) genes, even though in the mentioned phylogenetic tree, *A. nasomaculatus* nested within the clade of the genus *Oryx*, but *O. gazella* was distinguished from other species.

In the present paper, two different clades were introduced within the genus *Oryx*; this topology is proposed as follows:

[{(*O. dammah* +*O. leucoryx*)+*O. beisa*}+{*O. gazella*}]; and *A. nasomaculatus* as a sister group for this monophyletic group.

The genus *Hippotragus* consists of three species: *H. leucophaeus* (blaubok) (extinct), *H. equinus* (roan antelope), *H. niger* (sable). *H. n. roosevelti* (Roosevelt's sable) and *H. n. variani* are sometimes considered as full species (Groves and Grubb 2011). In the previous studies (Hassanin *et al*. 2012, Yang *et al*. 2013, Themudo *et al*. 2015), only complete sequences of mtDNA of *H. niger* and *H. equinus* were used, while in this study, complete mitochondrial genomes of Blaubok (*H. leucophaeus*) were

also compared. As a result, the topology of the phylogenetic relationships within this genus may be different.

Relatively, only very little has been published on the relationship between this extinct species (*H. leucophaeus*) and other species of the genus *Hippotragus*. The results of this study indicate that *H. n. niger* and *H. n. variani* have a very close phylogenetic relationship with the blaubok (*H. leucophaeus*) forming a single monophyletic group with highest bootstrap support value (=100). The roan antelope (*H. equinus*) is nested as their sister group with a high bootstrap value (=94). There is no complete mitochondrial sequence of *H. n. roosevelti or H. n. variani* Cytochrome c oxidase I phylogenetic tree also confirmed this topology, where two sequences of *H. niger* constructed a single monophyletic group with *H. leucophaeus* with a very high BI posterior probability value (=97.79), and *H. equinus* is the sister group of this clade. This topology has been also depicted by Themudo and Campos (2018).

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References

Aliabadian M, Nijman V, Mahmoudi A, Naderi M, Vonk R and Vences M. 2014. ExcaliBAR: a simple and fast software utility to calculate intra- and interspecific distances from DNA barcodes. *Contrib Zool*, **83(1):** 79-83.

Amir SA.M and Shobrak M. 2011. The efficiency of mitochondrial DNA markers in constructing genetic relationships among *Oryx* species. *Afr J Biotechnol.*, **10(22):** 4581-4585.

Arif IA, Khan HA, Bahkali AH, Al-Homaidan AA, Al-Farhan AH, Shobrak M and Al-Sadoo M. 2009. Comparison of Neighbor joining and maximum parsimony methods for molecular phylogeny of *Oryx* species using 12S rRNA and 16S rRNA gene sequences. *Animal Biol.*, **1(2):** 1-8.

Butynski TM, Parker I and de Jong YA. 2015. Historic and current distribution, abundance, and habitats of roosevelt's sable antelope *Hippotragus niger roosevelti* (Heller, 1910) (Cerartiodactyla: Bovidae) in Kenya. *J East Afr Nat Hist.*, **104(1&2):** 41–77.

Castelló J.R. 2016. **Bovids of the world: Antelopes, Gazelles, Cattle, Goats, Sheep, and Relatives**. Princeton University Press, Princeton, New Jersey, 664 pp.

Elmeer K, Almalki A, Mohran KA, Al-Qahtani KN and Almarri M. 2012. DNA barcoding of *Oryx leucoryx* using the mitochondrial cytochrome C oxidase gene. *Genet Mol Res.*, **11:** 539–547.

Excoffier L, Laval G and Schneider S. 2005. Arlequin ver. 3.0: an integrated software package for population genetics data analysis. *Evol Bioinform Online*, **1:** 47–50.

Felsenstein J. 1985. Confidence limits on phylogenies: An approach using the bootstrap. *Evol.*, **39:** 783-791.

Ghassemi-Khademi T. 2017. Evaluation of phylogenetic relationships of *Antilopini* and *Oreotragini* tribes (Bovidae: Artiodactyla) based on complete mitochondrial genomes. *J Wildlife and Biodiver.*, **1(1):** 1-11.

Ghassemi-Khademi T. 2018. New insight into the phylogeny of the orchid bees (Apidae: Euglossini). *J Wildlife and Biodiver.*, **2(1):** 19-35.

Groves C and Grubb P. 2011. **Ungulate Taxonomy**. Baltimore: John Hopkins Univsersity Press.

Geraads D, Blondel C, Likius A, Mackaye HT, Vignaud P and Brunet M. 2008. New Hippotragini (Bovidae, Mammalia) from the Late Miocene of Toros-Menalla (Chad). *J Vert Paleontol.*, **28:** 231–242.

Hall TA. 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symposium Series 41: 95–98. [London] Heckel, J.J. 1849. Die Fische Persiens gesammelt von Theodor Kotschy. *In* J. Russegger (Ed.), **Reisen in Europa**, Asien und Afrika (pp. 255-335). Volume 2. Stuttgart: Schweitzerbart'sche Verlagsbuchhandlung.

Hasegawa M, Kishino H and Yano T. 1985. Dating of human-ape splitting by a molecular clock of mitochondrial DNA. *J Mol Evol*, **22(2):** 160–174.

Hassanin A and Douzery EJ. 2003. Molecular and morphological phylogenies of Ruminantia and the alternative position of the Moschidae. *Syst Biol.*, **52:** 206–228.

Hassanin A, Delsuc F, Ropiquet A, Hammer C, Jansen van Vuuren B, Matthee C, Ruiz-Garcia M, Catzeflis F, Areskoug V, Nguyen T and Couloux A. 2012. Pattern and timing of diversification of Cetartiodactyla (Mammalia, Laurasiatheria), as revealed by a comprehensive analysis of mitochondrial genomes. *C R Biol.*, **335(1):** 32-50.

Holland W. 2015. Systematics of the Tribe Hippotragini (Cetartiodactyla: Bovidae). Emma Smith Hough Library Research Scholarship Awards, Southern Illinois University Carbondale (Open SIUC), ZOOL 405, 39 pp.

Huelsenbeck JP and Ranala B. 2004. Frequentist properties of Bayesian posterior probabilities of phylogenetic trees under simple and complex substitution models. *Syst Biol.*, **53:** 904-913.

Iyengar A, Diniz FM, Gilbert D and Woodfine J. 2006. Structure and evolution of the mitochondrial control region in *oryx*. *Mol. Phylogenetics Evol.*, **40:** 305-314.

Khan HA, Arif IA, Al-Farhan AH and Al-Homaidan AA. 2008a. Phylogenetic analysis of oryx species using partial sequences of mitochondrial rRNA genes. *Genet Mol Res.*, **7(4):** 1150-1155.

Khan HA, Arif IA, Shobrak M, Homaidan AAA, Farhan AHA and Sadoon MA. 2011. Application of mitochondrial genes sequences for measuring the genetic diversity of Arabian oryx. *Genes Genet Syst.*, **86:** 67–72.

Khan HA, Arif IA, Al-Homaidan AA and Al-Farhan AH. 2008b. Application of 16S rRNA, cytochrome b and control region sequences for understanding the phylogenetic relationships in oryx species. *Genet Mol Res.*,**7(4):** 1392-1397.

Khan HA, Arif IA, Bahkali AH, Al-Farhan AH and Al-Homaidan AA. 2008c. Bayesian, maximum parsimony and UPGMA models for inferring the phylogenies of Antelopes using mitochondrial markers. *Evol Bioinform.*, **4:** 263–270.

Kimura M. 1980. A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. *J Mol Evol.*, **16:** 111-120.

Kingdon J and Hoffmann M. 2013. **Mammals of Africa:** Vol. VI: Pigs, Hippopotamuses, Chevrotain, Giraffe, Deer and Bovids. Bloomsbury Publishing, London.

Kuznetsova MV, Kholodova MV and Luschekina AA. 2002. Phylogenetic analysis of sequences of the 12S and 16S rRNA mitochondrial genes in the family Bovidae: new evidence. *Russ J Genet.*, **38:** 942–950.

Krzywinski J, Grushko OG and Besansky NJ. 2006. Analysis of the complete mitochondrial DNA from *Anopheles funestus*: an improved dipteran mitochondrial genome annotation and a temporal dimension of mosquito evolution. *Mol Phylogenet Evol.*, **39:** 417–423.

Lydekker R and Blaine G. 1914. **Catalogue of the Ungulate Mammals in the British Museum (Natural History).** London: British Museum (Natural History).

Masembe C, Muwanika VB, Nyakaana S, Arctander P and Siegismund HR. 2006. Three genetically divergent lineages of the *Oryx* in Eastern Africa: Evidence for an ancient introgressive hybridization. *Conserv Genet.*, **7:** 551–562.

Matthee CA and Robinson T.J. 1999. Mitochondrial DNA population structure of roan and sable antelope: implications for

the translocation and conservation of the species. *Mol Ecol*., **8:** 227-238.

Matthee CA and Davis SK. 2001. Molecular insights into the evolution of the family Bovidae: a nuclear DNA perspective. *Mol Biol Evol*., **18:** 1220–1230.

Osmers B, Petersen BS, Hartl GB, Grobler JP, Kotze A, Van Aswegen E and Zachos FE. 2012. Genetic analysis of southern African gemsbok (*Oryx gazella*) reveals high variability, distinct lineages and strong divergence from the East African *Oryx Oryx beisa*. *Mamm. Mamm Biol*—Zeitschrift für Säugetierkd, **77:** 60–66.

Posada D and Crandall KA. 1998. Modeltest: testing the model of DNA substitution. *Bioinfor*., **14:** 817-818.

Price SA, Bininda-Emonds OR and Gittleman JL. 2005. A complete phylogeny of the whales, dolphins and even-toed hoofed mammals (Cetartiodactyla). *Biol Rev.*,**80:** 445–473.

Prothero DR and Foss SE. 2007. **The Evolution of Artiodactyls**. JHU Press.

Ronquist F and Huelsenbeck JP. 2003. MRBAYES 3: Bayesian phylogenetic inference under mixedmodels. *Bioinfor*., **19:** 1572-1574.

Ropiquet A. 2006. Etude des radiations adaptatives au sen des Antilopinae (Mammalia, Bovidae) PhD. Dissertation. Universite Paris 6.

Turner A and Anton M. 2004. **Evolving Eden: an illustrated guide to the evolution of the African large-mammal fauna**. Columbia university press.

Tamura K. 1992. Estimation of the number of nucleotide substitutions when there are strong transition-transversion and G + C-content biases. *Mol Biol Evol*., **9:** 678-687.

Tamura K, Stecher G, Peterson D, Filipski A and Kumar S. 2013. MEGA6: Molecular evolutionary genetics analysis, *Mol Biol Evol.*, **30:** 2725-2729.

Themudo GE, Rufino AC and Campos PF. 2015. Complete mitochondrial DNA sequence of the endangered giant sable antelope (*Hippotragus niger variani*): Insights into conservation and taxonomy. *Mol Phylogenetics Evol*., **83:** 242–249.

Themudo GE and Campos PF. 2018. Phylogenetic position of the extinct blue antelope, *Hippotragus leucophaeus* (Pallas, 1766) (Bovidae: Hippotraginae), based on complete mitochondrial genomes. *Zool J Linn Soc.*, **182(1):** 225-235.

Thompson JD, Higgins DG, Gibson TJ. 1994. CLUSTAL W: Improving the sensitivity of progressive multiple alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res*, **22:** 4673-4680.

Wilson DE and Reeder DM. 2005. **Antilopinae, in Mammal Species of the World: A Taxonomic and Geographic Reference**, Johns Hopkins University Press, 3ª ed.

Win NZ, Choi EY, Park J and Park JK. 2017. Molecular phylogenetic relationship of the subfamily Nymphalinae (Lepidoptera: Nymphalidae) in Myanmar, inferred from mitochondrialgene sequences. *J Asia Pac Biodiver*., **10:** 86-90.

Wright S. 1978. **Evolution and Genetics of Populations.** Chicago: University of Chicago.

Yang C, Xiang C, Qi W, Xia S, Tu F, Zhang X, Moermond T and Yue B. 2013. Phylogenetic analyses and improved resolution of the family Bovidae based on complete mitochondrial genomes. *Biochem. Syst. Ecol* **48:** 136–143.

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Cloning of the Organophosphorus Hydrolase (*oph)* Gene and Enhancement of Chlorpyrifos Degradation in the *Achromobacter xylosoxidans* Strain GH9OP via Mutation Induction

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Abstract

In this study, the *Achromobacter xylosoxidans* strain GH9OP showed an effective activity in chlorpyrifos degradation. This bacterial strain was able to degrade 34.72 % of chlorpyrifos compared to 1.03 % in the control media after five days. The *oph* gene of the *Achromobacter xylosoxidans* strain GH9OP, which encodes a protein involved in chlorpyrifos hydrolysis, was cloned and sequenced. It is a member of the MBL-fold metallo hydrolase superfamily, and has a beta lactamase fold. Moreover, it has 99 % similarity with that of *Achromobacter xylosoxidans* NH44784-1996. The DNA sequence of the open reading frame of the *oph* gene was deposited in Genbank database under accession number MH018244. AchM15 and AchMS1 were the best mutants in the chlorpyrifos biodegradation after the ethylmethane sulphonate (EMS) mutation induction for the *Achromobacter xylosoxidans* strain GH9OP resulting from first-step and second- step EMS mutation induction, respectively. AchM15 was able to degrade 63 % of chlorpyrifos compared to 34 % in wild type after five days. However AchMS1 exhibited the ability to degrade 82.03 % of chlorpyrifos compared with 65 % by AchM15 after five days. 3, 5, 6- trichloro-2-pyridinol (TCP) as a metabolite of the chlorpyrifos biodegradation, which was further metabolized to unknown polar metabolites, was detected using GC/MS after ten days in the case of AchMS1.

Keyword: *Achromobacter xylosoxidans*, Chlorpyrifos, Biodegradation, *oph*, Gene cloning, Ethylmethane sulphonate mutation.

1. Introduction

From the most important factors contributing to the massive increase in food production worldwide is the use of pesticides in agricultural systems. Moreover, applications of pesticides have been an environmental concern for the past several decades (Wang *et al.,* 2012) and their widespread use has caused environmental pollution (Lew *et al.,* 2013; Liu *et al.,* 2013). Organophosphate (OPs) compounds are highly potent neurotoxins that are commonly used as pesticides. These compounds inhibit acetylcholine esterase and disrupt the normal function of the central nervous system followed by severe muscle paralysis and death (Carvalho *et al.,* 2003). OPs are much more toxic to vertebrates compared to other of insecticide classes (Malhat and Nasr, 2011). Chlorpyrifos (O,O-diethyl O-(3,5,6-trichloro-2-pyridyl phosphorothioate), for instance, has been extensively applied in the household and for agricultural pest control. It is a non-systemic insecticide and acaricide with contact, ingestion, and inhalation (Abraham *et al.,* 2016; Thabit and El-Naggar, 2013).

A high concentration of chlorpyrifos was determined in Egyptian soils (Metwally, 2014). There are several methods available for the detoxification of these OPs including chemical treatment, incineration, volatilization, and photodecomposition, but most of them are not applicable for the complete removal of pesticide contamination at high concentrations because they are not environmental friendly, inefficient, and expensive. Microbial biodegradation is a reliable and costly-effective technology used for removing organophosphates (OPs) from surface water and soils (Chishti *et al*., 2013; Abraham and Silambarasan, 2016). Several bacterial isolates have been reported to be potent in the OPs biodegradation such as *Flavobacterium* sp. (Sethunathan and Yoshida, 1973), *Arthrobacter* sp. (Mallick *et al.,* 1999) and *Pseudomonas diminuta* (Serdar *et al.,* 1982). Chlorpyrifos biodegradation by soil bacteria, comprising seven different isolates of *Pseudomonas*, *Bacillus* and *Agrobacterium,* has been investigated (Maya *et al.,* 2011).

Mutagenesis through classical genetic approaches involves the use of random mutations to improve the desired metabolites' yields and the bacterial enzymes' production, in particular, bacterial wild strains (Chen *et al.,* 2008). The advantages of random mutagenesis using

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chemical mutagens such as ethyl methyl sulphonate (EMS) are more overwhelming due to their simplicity and low cost procedures compared to DNA recombinant (Munazzah *et al.*, 2012; Rowland, 1984).

Organophosphorus hydrolase (OPH) enzyme are encoded by *oph* gene, *mpd* gene and/or *opd* gene in different unrelated bacterial strains. These genes have no a DNA- sequence similarity (Bigley and Raushel, 2013).

The objectives of this study are to identify and characterize the *oph* gene responsible for chlorpyrifos biodegradation in the *Achromobacter xylosoxidans* strain GH9OP. Improving the ability of this strain for chlorpyrifos biodegradation by chemical mutation induction using ethylmethane sulphonate (EMS) is the second goal of this research.

2. Materials and Methods

2.1. Reagents and Chemicals

Analytical grade chlorpyrifos (48 %) emulsifiable concentrate was purchased from (Sinochem Agro.Co.Ltd, China). All reagents and solvents used in the present study were of the analytical grade. Chlorpyrifos was used as an organophosphorus insecticide model due to its high residues in Egyptian soils (Metwally, 2014).

2.2. Evaluation the Chlorpyrifos Biodegradation by Achromobacter xylosoxidans Strain GH9OP

The *Achromobacter xylosoxidans* strain GH9OP isolated and identified in microbial genetic department, national research Centre, was friendly taken and inoculated in broth minimal salt media (Fang *et al.*, 2008) containing chlorpyrifos with a concentration of 480 mg/L as a sole carbon source and was incubated on an orbital shaker (Thermoscientific, UK) at 30 ^{o}C for five, ten, and fifteen days at 150 rpm, and samples were done in triplet (Singh and Walker, 2006). At the same time, non-inoculated media were also run in parallel to the other cultures as control. The extraction of chlorpyrifos residues was done as follow: a known volume of a mineral salt liquid media (MSL), 100 mL, was transferred into a 500 mL separatory funnel, and was partitioned successively three times with 50 mL of dichloromethane each and 40 mL of a sodium chloride solution (20 %). The combined extracts were filtered through a pad of cotton and anhydrous sodium sulfate, and were then evaporated at 30 ^{o}C to dryness using a rotary evaporator at 30^{o}C. After that, the residue was quantitatively transferred to a standard glass stopper test tube with ethyl acetate, and the solvent was evaporated to dryness (Metwally, 2014). The Chlorpyrifos residue and its metabolites were determined using Gas Chromatography analysis in the Central Agriculture Pesticides Lab (CAPL), Agriculture Research Center, Giza, Egypt. At the same time, 1 mL of the bacterial culture was used to make serial dilutions 10^{-4} to 10^{-6} and was plated on Luria-Bertani (LB) agar plate. The plate was incubated at 30 ^{o}C for eighteen hours, and a single colony was subjected to DNA extraction and EMS mutation induction.

2.3. Data Calculation

$$\text{Degradation (\%)} = \left(\frac{\text{Residual amount in blank control} - \text{Residual amount in sample}}{\text{Residual amount in blank control}}\right) \times 100$$

2.4. Extraction of Genomic DNA from the Achromobacter xylosoxidans Strain GH9OP

A Single colony was cultured in a conical flask (Pyrex, USA) containing 20 mL of an LB medium by shaking in an orbital shaker (Thermo fisher scientific, UK) at 150 rpm for eighteen hours. The culture was centrifuged at 13,000 rpm for five minutes at 4^{o}C. The pellet was subjected to genomic DNA extraction using the (QIAamp DNA Mini Kit, QIAGEN, Germany). The extracted DNA was used as a template for PCR to amplify the *oph* gene.

2.5. Cloning and Sequencing of the oph Gene: The Gene That Might Be Responsible for Chlorpyrifos Degradation in the Achromobacter xylosoxidans Strain GH9OP

Sequence of the *oph* gene in *Achromobacter xylosoxidans* GH9OP was predicted based on search results from the conserved domain database (CDD) of NCBI, The *oph* gene of putative organophosphorus hydrolase (oph) enzyme was detected by the analysis of the complete genomic sequence of the *Achromobacter xylosoxidans* strain NH44784-1996 genbank HE798385 region, 1841224 to 1842159. PCR amplification of the *oph* gene by the sense primer F-oph 5´GGGGATCCATGCCATCCGCAACCCAAACCC´3 and antisense primer R-oph 5´GGAAGCTTTCAGTAGTCCCATATGACCGGC´3 using the *Achromobacter xylosoxidans* strain GH9OP genomic DNA as template, was conducted with GeneAmp PCR system 400 thermal cycler (PerkinElmer, Norwalk, Connecticut, USA). F-oph and R-oph primers were designed to contain, respectively, a *Bam* HI restriction site at *oph* start codon and *Hind* III restriction site at the stop codon (underlined bases) to facilitate cloning. In this reaction, amplification was carried out in a 50 µL reaction mixture by using a PCR master mix kit (Qiagen, Germany) according to the manufacturer's instructions. The following program was used: 94^{o}C for three minutes as the initial denaturation step, followed by thirty-five cycles of denaturation at 94^{o}C for thirty seconds, annealing at 55^{o}C for one minute and extension at 72°C for one minute, and a final extension step at 72^{o}C for ten minutes. The anticipated PCR product of 950 bp was isolated after agarose gel electrophoresis using a gel extraction kit (Qiagen, Germany). The amplified fragment was cloned into the pTZ57R/T cloning vector (Thermo Scientific, Germany), and was transformed into *E. coli* XL1-Blue. Standard procedures for restriction endonuclease digestions, agarose gel electrophoresis, DNA ligation, and other cloning- related techniques were used as described by Sambrook and Russell (2001). Plasmids from transformant colonies were purified using Mini Plasmid Kit (Qiagen, Germany). The purified recombinant vector was sent to Clinilab, in Egypt for sequencing using vector primers. The obtained sequence was compared to other known sequences found in the Genbank by using the Blast program (http://www.ncbi.nlm.nih.gov/BLAST/)

2.6. Ethylmethane Sulfonate (EMS) Mutation Induction of the Achromobacter xylosoxidans strain GH9OP and Mutant Selection

The first-step EMS mutation induction was done as follows: one ml of an eighteen-hour old culture was centrifuged at 12,000 rpm for five minutes. The pellet was washed with and dissolved in 1mL of a 100 mM sodium phosphate buffer pH 7. 100 µL of the sample content was withdrawn to determine the initial population (CFU/mL). 20 µL/mL of the EMS stock solution 1gm/ml (Merck) was added to the samples in the falcon tubes for different times twenty, forty and sixty minutes, and was then incubated at 30°C. The reaction was stopped by the addition of 4 mL of sodium thiosulfate (5 %). The reaction was centrifuged; the pellets were washed and resuspended in a sodium phosphate buffer. Portions of 0.1 mL of suitable dilutions were spread on LB agar plates and incubated at 30°C for forty-eight hours. The bacterial colonies developed after incubation were counted and the survival percentages were estimated for each treatment (Verma *et al.*, 2016). Second-step EMS mutation induction was employed for the best mutant which resulted from first-step EMS mutation induction in the same manner illustrated earlier. In this study, the results of improved mutants are directed primarily based on their potential in the chlorpyrifos biodegradation compared to their wild strain.

3. Results

3.1. Evaluation of Chlorpyrifos Biodegradation by the Achromobacter xylosoxidans Strain GH9OP

Achromobacter xylosoxidans strain GH9OP showed effective activity in chlorpyrifos degradation. Figure 1 shows chlorpyrifos biodegradation by the *Achromobacter xylosoxidans* strain GH9OP after five, ten, and fifteen days; this bacterial strain was able to degrade 34.72 % after five days and 79.43 % after fifteen days of chlorpyrifos compared 1.03 % after five days and 3.05 % after fifteen days in the control media.

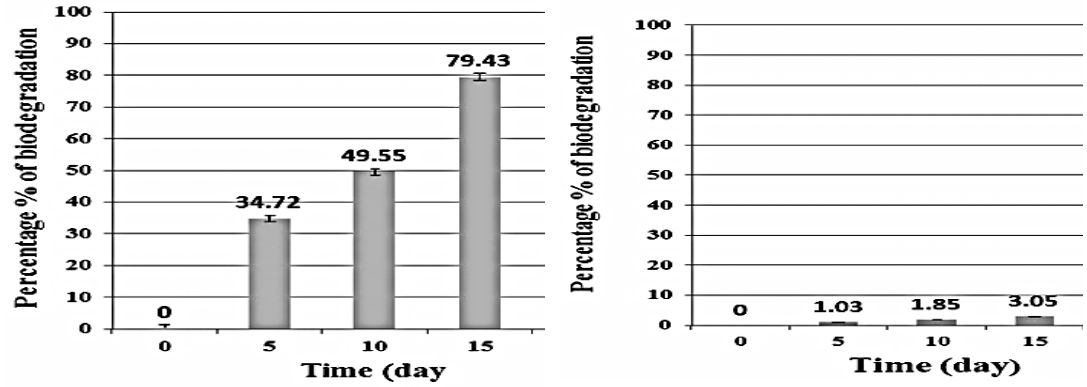

Figure 1. Biodegradation percentage of chlorpyrifos (480 mg/l) by *Achromobacter xylosoxidans* strain GH9OP (A). Degradation percentage of chlorpyrifos (480 mg/L) in control media (B).

3.2. Cloning and Complete Sequencing of the oph Gene in the Achromobacter xylosoxidans strain GH9Op

Primers, F-oph and R-oph containing *Bam* HI and *Hind* III restriction sites were used to amplify the complete coding sequence of the *oph* gene with a size of ~ 950 bp (Figure 2).

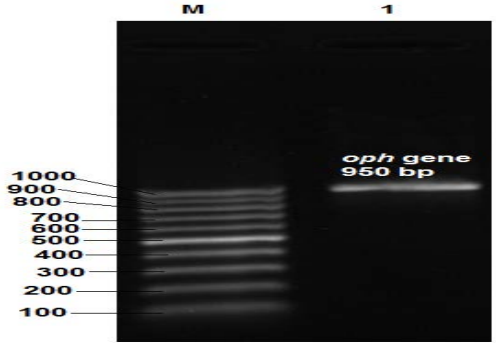

Figure 2. Agarose gel electrophoresis of PCR amplification for the oph gene in the Achromobacter xylosoxidans strain GH9OP (1). M: 100 bpDNA ladder (Jenabio).

The amplified fragment was cloned into pTZ57R/T cloning vector, and transformed into *E. coli* XL1-Blue. The new construct of pTZ57R/T vector carrying *oph* was termed as pTZ57R-oph.

Screening of transformant colonies with the recombinant vector and those containing non-recombinant vector was estimated by rapid screening of Plasmids using plasmid mini prep (Qiagen, Germany) (Figure 3), colony pcr using F-oph and R-oph (Figure 4) and Double digestion of pTZ57R-oph using *Bam* HI and *Hind* III endonuclease (Figure 5).

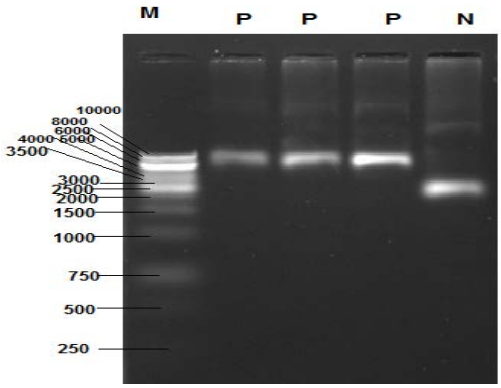

Figure 3. Agarose gel electrophoresis of isolated plasmid from transformant E. coli XL1-Blue. P: transformant colonies containing pTZ57R-oph vector. N: transformant colonies containing pTZ57R vector only.

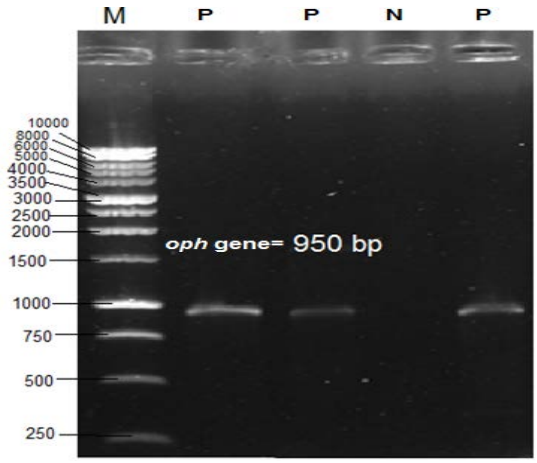

Figure 4. Agarose gel electrophoresis for colony PCR products of three colonies of *E. coli* XL1-Blue transformed with the recombinant plasmid pTZ57R-oph containing *oph* gene from *Achromobacter xylosoxidans* strain GH9OP by using *oph* gene primers. (M): 1 Kb DNA ladder (Jenabio). P: Positive colony in pcr amplification of *oph* gene. N: Negative colony in PCR amplification of *oph* gene.

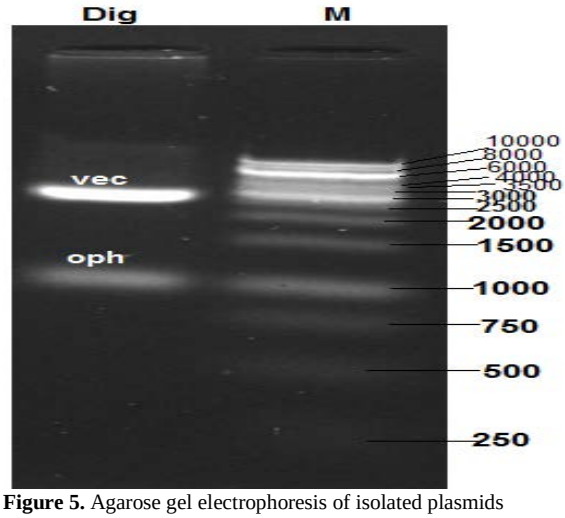

Figure 5. Agarose gel electrophoresis of isolated plasmids digested by *Bam* HI and *Hind* III restriction enzymes (Dig). (M): I Kb DNA ladder (Jenabio).

The DNA sequence of the *oph* gene was subjected to a search on (https://www.ncbi.nlm.nih.gov/ Structure/ cdd/ wrpsb.cgi), and the result was that a conserved domain in the isolated *oph* gene is a member of the MBL-fold metallo hydrolase superfamily and has a beta lactamase fold. It has a 99 % similarity with that of *Achromobacter xylosoxidans* NH44784-1996 Accession no. HE798385.1. Deduced protein sequences of both genes were aligned using CLUSTAL multiple sequence alignment, MUSCLE 3.8 (Figure 6). The *oph* gene in *Achromobacter xylosoxidans* GH9OP was firstly isolated by the authors of this study from Egyptian soils, and the DNA sequence of the *oph* gene was deposited in Genbank database under the accession number MH018244.

```
MH018244.1                MPSATQTLPFSSLSDPCVRPPHDLVPSRYALRVGEIDALVISDGVLPLPT
HE798385.1_1841224-1842159 MPSATQTLPFSSLSDPGVRPPHDLVPSRYALRVGEIDALVISDGVLPLPT
                          *************** ************************************

MH018244.1                ATMATNADPADLARWLQYMFMPPDAFDWPLNVMVARSGDQTILIDAGLGG
HE798385.1_1841224-1842159 ATMATNADPADLARWLQYMFMPPDAFDWPLNVMVARSGDQTILIDAGLGG
                          ************************************************

MH018244.1                QFPGFPRAGQLPQRLEDAGIALESVTDVIITHMHMDHVGGLLVDGVKERL
HE798385.1_1841224-1842159 QFPGFPRAGQLPQRLEDAGIALESVTDVIITHMHMDHVGGLLVDGVKERL
                          ************************************************

MH018244.1                RPDVRIHVSATEVAFWTSPDFSHTVMPKPVPAVLRSTAASFYNEYRDRLR
HE798385.1_1841224-1842159 RPDVRIHVSATEVAFWTSPDFSHTVMPKPVPAVLRSTATSFYNEYRDRLR
                          ************************************:***********

MH018244.1                IFQDRHEVAPGVVVRITGGHTPGHSVVDLIGGDERLTFAGDAIFPVGFDH
HE798385.1_1841224-1842159 IFQDRHEVAPGVVVRITGGHTPGHSVVDLIGGDERLTFAGDAIFPVGFDH
                          ************************************************

MH018244.1                PDWHNGFEHDPEESARVRLRLFQELAQNRGLLVAAHLPFPSVGRVAIDGD
HE798385.1_1841224-1842159 PDWHNGFEHDPEESARVRLRLFQELAQNRGLLVAAHLPFPSVGRVAIDGE
                          ************************************************:

MH018244.1                AFRWVPVIWDY
HE798385.1_1841224-1842159 AFRWVPVIWDY
                          ***********
```

Figure 6. Pairwise alignment of deduced amino acid sequences of the *oph* gene from the *Achromobacter xylosoxidans* strain GH9OP accession no. MH018244 and the organophosphorus hydrolase enzyme (*oph)* gene from *Achromobacter xylosoxidans* NH44784-1996 Accession no. HE798385.1:1841224-1842159.

3.3. EMS Mutation Induction in Achromobacter xylosoxidans GH9Op

3.3.1. First-Step EMS Mutation Induction in Achromobacter xylosoxidans GH9Op

The Mutagenesis approach was widely used to improve the production of various microbial enzymes (Hussein *et al.*, 2012). It is the first time to improve the organophosphorus hydrolase enzyme responsible for chlorpyrifos biodegradation by random mutation using ethyl methanesulfonate (EMS) as a chemical mutagen. After the induction of EMS mutation with the *Achromobacter xylosoxidans* strain GH9OP for different treatment periods, viable colonies were counted from appropriate dilution. The survival percentage was calculated and the results are presented in Figure 7. It was noticed that the survival percentage decreased as the treatment time increased due to the lethal effect of EMS. The least survival percentage was recorded at an exposure period of forty minutes. The exposure period of sixty minutes was lethal for the wild strain.

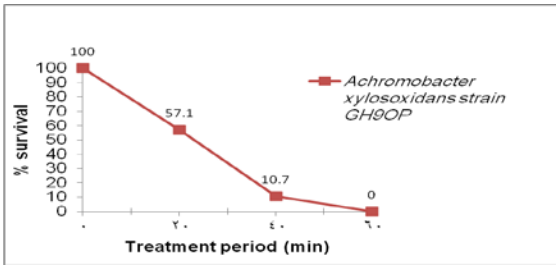

Figure 7. Survival percentage of *Achromobacter xylosoxidans* strain GH9OP after EMS (20 µL/mL) mutation induction.

3.3.1.1. Effect of EMS Mutation Induction on the Biodegradation of Chlorpyrifos by Achromobacter xylosoxidans GH9OP

In spite of the fact that genetic engineering has made a significant contribution to the improvement of bacterial strains, random mutagenesis is still a cost-effective procedure for reliable short-term strain development and is frequently preferred as the method of choice (Lipika, 2014). EMS is a well-known chemical mutagenic agent whose mode of action is attributed to the alkylation of nitrogen position 7 of guanosine of the DNA molecule causing G/C to A/T transition mutation (Freese, 1961).

After a twenty-minute treatment by EMS, twenty-nine mutants were selected, twenty-one mutants exhibited approximately equal activity to the wild type, and eight mutants exhibited higher activity compared to the wild type. The highest biodegradation was recorded by AchM12 and AchM15 which were able to degrade 63 % of chlorpyrifos compared to 34 % in the wild type. After forty minutes of the EMS treatment, sixteen mutants were selected; seven mutants exhibited less activity than the wild type, three mutants exhibited approximately equal activity to the wild type, and only four mutants exhibited a higher activity than the wild type especially AchM44 which degraded 49 % chlorpyrifos. All biodegradation activity was recorded after five days (Table 1) .

Table 1. Chlorpyrifos biodegradation by EMS-induced mutants from the *Achromobacter xylosoxidans* strain GH9OP after five days

Treatment exposure time (min)	Bacterial isolates	*dry weight g/100 mL	*% chlorpyrifos biodegradation	Bacterial isolates	*dry weight g/100 ml	*% chlorpyrifos biodegradation
20	wild type	0.281	34.23	AchM15	0.289	63.41
	AchM1	0.290	33.90	AchM16	0.269	34.08
	AchM2	0.271	33.90	AchM17	0.290	34.01
	AchM3	0.273	34.50	AchM18	0.289	34.90
	AchM4	0.254	36.89	AchM19	0.278	41.95
	AchM5	0.270	33.99	AchM20	0.301	33.99
	AchM6	0.291	36.07	AchM21	0.258	33.98
	AchM7	0.287	37.13	AchM22	0.294	37.91
	AchM8	0.279	33.99	AchM23	0.290	36.93
	AchM9	0.269	33.98	AchM24	0.286	33.00
	AchM10	0.269	34.09	AchM25	0.293	34.08
	AchM11	0.291	34.08	AchM26	0.310	37.30
	AchM12	0.276	63.20	AchM27	0.297	34.70
	AchM13	0.293	33.89	AchM28	0.311	34.70
	AchM14	0.287	33.44	AchM29	0.264	34.49
40	AchM30	0.201	33.97	AchM38	0.08	1.13
	AchM31	0.253	36.90	AchM39	0.188	19.80
	AchM32	0.290	34.09	AchM40	0.299	42.00
	AchM33	0.281	34.10	AchM41	0.190	14.97
	AchM34	0.063	0.93	AchM42	0.289	42.98
	AchM35	0.191	15.09	AchM43	0.179	22.13
	AchM36	0.189	21.50	AchM44	0.301	49.00
	AchM37	0.193	20.69	AchM45	0.180	18.16

*Mean of three replicates

3.3.2. Second-Step EMS Mutation Induction

The highest mutant, AchM15, in the chlorpyrifos biodegradation, resulted from the first-step EMS mutation induction for the *Achromobacter xylosoxidans* strain GH9OP for twenty minutes which was then mutated by EMS under the same conditions previously employed for the wild bacterial isolates. The highest mutant (AchMS1) was obtained after the second-step EMS treatment of AchM15 for forty minutes; it recorded a higher biodegradation potential than AchM15. It degraded 82.03 % of chlorpyrifos compared with 65 % by AchM15 after five days.

This study achieved improved OPH (organophosphorus hydrolase) variants with increased activity towards poorly-hydrolysed substrates such as chlorpyrifos by random mutation using the EMS-treatment approach which leads to an enzymatic solution. Figure 8 shows the growth of the wild type, *Achromobacter xylosoxidans* strain GH9OP and its mutants, AchM15 and AchMs1. All were grown in minimal media containing chlorpyrifos with the aforementioned concentration.

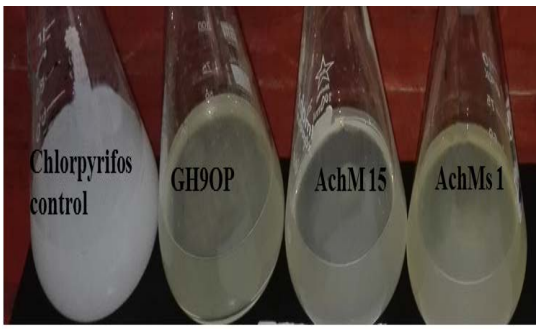

Figure 8. Bacterial growth of the *Achromobacter xylosoxidans* strain GH9OP and its mutants on liquid MSM containing Chlorpyrifos (480 mg/l) after 5 days. GH9OP: *Achromobacter xylosoxidans* wild strain. AchM15: mutants from first-step EMS mutation induction. AchMs1: mutant from second-step EMS mutation induction. Chlorpyrifos Control: liquid MSM containing chlorpyrifos without bacterial inoculum.

3.3.3. Evaluation of the Behavior of Achromobacter xylosoxidans GH9OP and Their Higher Mutants in OPs Degradation through Time Shift

The biodegradation of chlorpyrifos by *Achromobacter xylosoxidans* GH9OP as a wild strain and its selected higher mutants, AchM15 and AchMS1 were identified after five, ten, and fifteen days. Figure 9 shows that the periods of five and ten days were the perfect periods at which the highest biodegradation occurred in both wild bacterial strain and its mutants. These periods were the transformation points at which difference between wild strains and their mutants was emphasized. The *Achromobacter xylosoxidans* strain GH9OP degraded 54.22 % of chlorpyrifos and its mutants. AchM15 and AchMs1 degraded 75.08 % and 92.03 %, respectively compared with 7.14 % in case of control after ten days. It can be included that the biodegradation achieved by the wild strain over fifteen days could be achieved in five days by their higher mutants. All treatments were carried in triplicate.

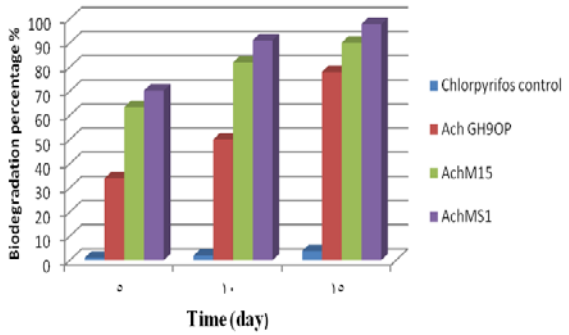

Figure 9. Chlorpyrifos biodegradation by *Achromobacter xylosoxidans* and its mutants**.**

3.4. Identification of the Degradation Products of Chlorpyrifos in Achms1 by GC/MS

In this study, it is the first time to manipulate the bacterial mutant, AchMS1, in pesticides' biodegradation so that it can be used as a model strain to detect chlorpyrifos metabolites. The metabolic products of chlorpyrifos degradation were confirmed by GC-MS. The results were based on the characteristic fragment ion peaks and molecular ion m/z 197. The new peak was identified as 3, 5, 6-trichloro-2-pyridinol (TCP) as the main metabolite of chlorpyrifos at a retention times (RT) of 30.03 minutes. This peak disappeared concomitantly with the formation of other new peaks with a retention time of around 24.1 minutes (Figure 10). Each peak was identified on the basis of its mass spectra and the NIST library identification program. The degradation pathway for chlorpyrifos by AchMS1 was proposed in Figure 11. That is to say, the parent chlorpyrifos (m/z = 351) was first metabolized by hydrolysis to produce TCP (m/z = 197) and diethyl thiophosphoric acid (DETP) (m/z = 172). Subsequently, the hydrolysis product, TCP was further transformed by a ring breakage, resulting in its detoxification. These results indicated that chlorpyrifos (480 mg/L) was degraded by AchMs1 without any accumulative products after ten days of incubation.

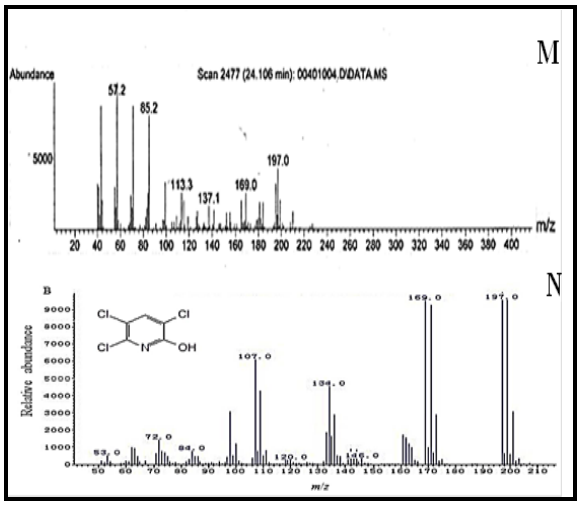

Figure 10. Mass spectra of 3, 5, 6-trichloro-2-pyridinol (TCP) produced from chlorpyrifos degradation by AchMS1. M: sample; N: authentic standard TCP from the National Institute of Standards and Technology (NIST, USA) library database.

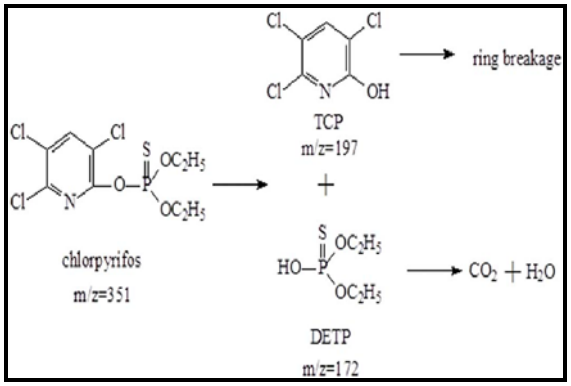

Figure 11. The proposed pathway for the chlorpyrifos biodegradation by AchMS1.

4. Discussion

The *Achromobacter xylosoxidans* strain, GH9OP, showed a high potential in chlorpyrifos biodegradation, This result is inconsistent with the previous reports that employed the *Achromobacter xylosoxidans* (JCp4) and *Ochrobacterum sp.*(FCp1) in chlorpyrifos biodegradation. It was found that these strains were able to degrade chlorpyrifos in non-sterilized and sterilized soils and exhibited the ability to degrade 93 % to 100 % of the input concentration 200 mg/l within forty-two days (Akbar and Sultan, 2016). In this study, the *Achromobacter xylosoxidans* strain GH9OP is a promising candidate for raising the productivity of crops in pesticide- contaminated soils. Many bacterial strains are involved in organophosphorus pesticides' degradation. Among these bacteria are: *Enterobacter* sp. (Singh *et al.*, 2004), *pleismonas* sp. (Zheng *et al.*, 2013), *Agrobacterium radiobacter* (Horne *et al.*, 2002), and *Streptomyces* sp. (Nelson, 1982). Different bacterial genes are responsible for the organophosphates degradation, and these genes exhibit different DNA sequences in different bacterial strains. For example, Serdar *et al.* (1982) isolated the first described organophosphorus degrading the *opd* gene from *P. diminuta,* and Mulbry *et al.* (1987) isolated the *opd* gene from the *Flavobacterium* sp. strain, ATCC 27551.

Significant homology between *opd* genes from the two bacteria was demonstrated by Southern hybridization experiments (Mulbry *et al.*, 1987). *opa*A gene is another organophosphorus-degrading gene which has received considerable attention. It was isolated and cloned from *Alteromonas* sp. JD6.5 by Cheng *et al.* (1997). Despite, functional similarity with the *opd* gene, no sequence homology was found between them. Zhongli *et al.* (2001) isolated a methyl parathion degrading the *mpd* gene from the *Plesiomonas* sp. strain M6. The sequencing and cloning of this gene revealed that no region of extensive DNA homology was observed between the *mpd* gene and those in the Genbank database. A 31 % similarity between protein sequence and beta-lactamase was found, suggesting the significant novelty of the gene-enzyme system.

According to the analysis of DNA sequence of the *oph* gene in the *Achromobacter xylosoxidans* GH9OP by using conserved domains on NCBI, (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi), this gene is a member of the MBL-fold metallo hydrolase superfamily, and has a beta lactamase fold which is mainly hydrolytic enzymes involved in the organophosphates hydrolysis (Bigley and Raushel, 2013). Salman *et al.* (2010) reported that a total of sixty different enzymes involved in organophosphate degradation are divided into eight subgroups based on the type of organism. No significant homology is observed overall, and poorly aligned regions at the ends of proteins can be easily seen. Moreover the active sites in all these enzymes do not possess conserved amino-acid sequences. Consequently, they give rise to a different active site structure.

Site-directed mutagenesis as a tool to manipulate Catalytic mechanism and enzyme properties was successfully used to enhance the activity of OPH against racemic mixtures of organophosphorus enantiomers. Wu *et al.* (2001) and Raushel, (2002) used site-directed mutagenesis for remoulding the size and shape of the substrate binding subsites through rational restructuring. However, due to unexpected influences exerted by substituted amino acids, rational designs can fail sometimes. It is the first time in this study to use EMS-mutation induction as an effective and cost-effective technique to improve the bacterial organophosphorus hydrolase enzyme activity towards poorly degradable substrates such as chlorpyrifos. The obtained higher mutants such as AchM15 and AchM12 exhibited a cell mass that was slightly equal to that of the wild type; this means that the increase in the biodegradation activity is related to the increase in the gene-enzyme expression. In the highest mutants, the increase in the chlorpyrifos biodegradation may be attributed to several reasons including the overexpression of the gene encoding the enzyme (organophosphorus hydrolase enzyme) responsible for the OPs biodegradation, which increases the efficiency of the secretion system. Changes in one or more amino acids of the enzyme result in more binding efficiency between the produced enzymes and their substrates leading to more biodegradation activity. EMS is able to induce the overproduction of other enzymes including α-amylase, xylanase, cellulase and laccase indifferent bacterial strains. When Haq *et al.* (2009) treated *Bacillus licheniformis* with EMS to improve the α-amylase production, the best mutant increased was by about 39 % compared to the wild type Strain. Hanim *et al.* (2013) improved xylanase production from the xylanolytic bacteria using EMS, and the specific activity was increased by 61.61 % from 9.317 U/g to 15.057 U/g. Narasimhan *et al.* (2013) used EMS to improve the cellulase activity of *Bacillus subtilis*, and mutant M57 showed 4.71 fold increasing in cellulase activities. Verma *et al.* (2016) employed EMS to improve the the production of laccase from *Pseudomonas putida*, LUA15.1, isolated from rice rhizospheric soil samples of paddy fields, in Himachal Pradesh (India). They obtained mutant E4 (34.12 U/L) resulting in a 26.37 % increase in the laccase activity compared to the wild strain (25.12 U/L). However, mutants, AchM34 and AchM38 showed no biodegradation activity, and this may be attributed to the damage of the structure gene where mutation changes in the structure genes most probably result in the loss of function (Ho and Chor, 2015). Their weak growth in the minimal media containing chlorpyrifos as a carbon source was due to their inability to produce the organophosphorus hydrolase enzyme responsible for the chlorpyrifos biodegradation and unused produced metabolites as a carbon source.

Vijayabaskar *et al.* (2014) used the best UV mutant of the *Bacillus pumilus* strain to further improve the Carboxy methyl cellulase by EMS. This treatment improved the activity by 96.24 %. Taking the same path, this study obtained AchMS1 from the EMS mutation second-step for AchM15. The increase in the biodegradation potential in AchMS1 may be attributed to stabilizing the structure of the enzyme and promoting the interactions between the enzyme and its substrate. Moreover, when a microbial culture is exposed simultaneously to mutagenic agents, the frequency of mutation increases. The duplication of the gene that occurred enables it to increase the inefficient enzyme production. As a result, mutations may occur in the enzymes' structural gene, enabling the former and inefficient enzymes to metabolize their substrates more efficiently and effectively (Monroe, 2005). It can be concluded that chemical mutagenic agents that possess the ability to induce mutation have been a major driving force in the field of genetic studies for the past seventy-five years (Muller, 1930).

This study generated potent OPs-degrading bacterial mutants contributing to increasing in OPS detoxification. AchMS1 exhibited the ability to degrade chlorpyrifos into non-polar compounds. This feature is rarely reported in other chlorpyrifos-degrading microorganisms. In most cases reported to date, the individual isolate is able to transform chlorpyrifos by hydrolysis of ester linkage to give TCP, which in turn accumulates in the batch cultures or soils. Moreover, enhanced degradation could not occur due to its antimicrobial properties (Anwar *et al.*, 2009; Li *et al.*, 2010). TCP has an estrogenic activity and has recently been listed as a potential endocrine disrupting chemical by the Environmental Protection Agency (EPA, 2009) of the USA. Compared with the importance of TCP degradation issue, studies concerning its degradation and fate in the environment are very limited. Chen *et al.* (2012) isolated a new fungal strain Hu-01 identified as *Cladosporium cladosporioides* with a high chlorpyrifos-degradation activity and utilized 50 mg/l of chlorpyrifos as the sole carbon source at acidic pH 6.5. This study is in contrast with his previous findings, because chlorpyrifos biodegradation by the bacterial mutant in this study was

perfect in a basic pH that is already present in most Egyptian agriculture soils. Another important feature worth mentioning is that the bacterial mutant under study engaged in efficient degradation of chlorpyrifos at a high concentration in contrast to other reports on the toxic effects of OPs in diverse microorganisms (Racke *et al.*, 1990; Mallick *et al.*, 1999). The high chlorpyrifos tolerance and degradation capability of the bacterial mutant make this mutant suitable for decontamination and bioremediation of the Ops- contaminated sites.

5. Conclusion

The present study investigated the ability of the *Achromobacter xylosoxidans* strain GH9OP in chlorpyrifos biodegradation. This strain exhibited a high chlorpyrifos biodegradation activity. The *oph* gene encoding the organophosphorus hydrolase enzyme responsible for chlorpyrifos biodegradation was cloned, and the ORF of this gene was sequenced for the first time. EMS mutation induction is an effective approach in the enhancement of the chlorpyrifos biodegradation activity. This study confirms the safe application of the AchMS1 bacterial mutant in chlorpyrifos bioremediation issues due to the production of metabolites which are less toxic than the parent substrate.

References

Abraham J and Silambarasan S. 2016. Biodegradation of chlorpyrifos and its hydrolysis product 3,5,6-trichloro-2-pyridi-nol using a novel bacterium *Ochrobactrum* sp.JAS2: a roposal of its metabolic pathway. *Pestic Biochem Physiol*, **126**:13–21.

Abraham J, Shanker A and Silambarasan S. 2013. Role of *Gordonia* sp. JAAS1 in biodegradation of chlorpyrifos and its hydrolysing metabolite 3,5,6-trichloro-2-pyridinol. *Lett Appl Microbiol*, **57**: 510–516.

Akbar S. and Sultan S. 2016. Soil bacteria showing a potential of chlorpyrifos degradation and plant growth enhancement. *Braz J Microbiol*, **47**: 563–570.

Anwar S, Liaquat F, Khan QM, Khalid ZM and Iqbal S. 2009. Biodegradation of chlorpyrifos and its hydrolysis product 3,5,6-trichloro-2-pyridinol by *Bacillus pumilus* strain C2A1. *J Hazard Mater*, **168**: 400–405.

Bigley AN and Raushel FM. 2013. Catalytic mechanisms for phosphotriesterases.*Biochim Biophys Acta*, 1**834**: 443-53.

Carvalho FD, Machado I, Martınez MS, Soares A and Guilhermino L. 2003. Use of atropine-treated Daphnia magna survival for detection of environmental contamination by acetylcholine esterase inhibitor. *Ecotoxicol Environ Saf*, **53**: 43–46.

Chen H L, Wan H G, Zhang J and Zeng X X. 2008. Mutation-screening of *Pleurotus ferulae* with high temperature tolerance by nitrogen ion implantation. *Plasma Sci Tech*, **10**: 503-505.

Chen S, Liu C, Peng C, Liu H, Hu M and Zhong G. 2012. Biodegradation of chlorpyrifos and its hydrolysis product 3,5,6-trichloro-2-pyridinol by a new fungal strain *Cladosporium cladosporioides* Hu-01. *PLoS ONE*, **7**: 1-12.

Cheng TC, Rastogi VK, DeFrank JJ, Anderson DM and Hamilton AB. 1997. Nucleotide sequence of a gene encoding and organophosphorus never agent degrading enzyme from *Alteromonas haloplanktis*. *J Ind Microbiol Biotechnol*, **18**: 49–55.

Chishti Z, Hussain S, Arshad KR, Khalid A and Arshad M. 2013. Microbial degradation of chlorpyrifos in liquid media and soil. *J Environ Manag*, **114**: 372–380

EPA, Washington, DC. 2009. Endocrine Disruptor Screening Program (EDSP). USA.

Fang H, Xiang YQ, Hao YJ, Chu XQ, Pan XD, Yu JQ and Yu YL. 2008. Fungal degradation of chlorpyrifos by *Verticillium* sp. DSp in pure cultures and its use in bioremediation of contaminated soil and pakchoi. *Inter Biodeterio Degradation*, **61**: 294-303.

Freese EB. 1961. Transitions and transversion induced by depurinating agents. *Proc National Academy of Sci USA*, **47**: 540-545.

Hanim C, Yusiati LM, Cahyanto MN and Wibowo A. 2013. Mutagenic improvement of xylanase production from xylanolytic bacteria and its phylogenetic analysis. *Microbiol. Indones*, **7**: 51-58.

Haq I, Ali S, Saleem A and Javed MM. 2009. Mutagenesis of *Bacillus licheniformis* through ethylmethane sulfonate for alpha amylase production. *Pakistan J Bot*, **41**: 1489-1498.

Ho LL and Chor XK. 2015. Improvement of Xylanase Production by *Bacillus subtilis* in Submerged Fermentation after UV and Chemicals Mutagenesis. *JABB*, **3**: 42-57.

Horne I, Suther TD, Harcourt RL, Russell RJ.and Oakeshott JG. 2002. Identification of an opd (organophosphate degradation) gene in an *Agrobacterium* isolate. *Appl Environ Microbiol*, **68**: 3371–3376.

Hussein AA, Ali NA and Jasim HM. 2012. Effect of physical and chemical mutagenesis on the ability of locally isolated *Bacillus stearothermophilus* in protease production. *Iraqi J Biotech*, **11**: 261-269.

Lew S, Lew M, Biedunkiewicz A and Szare J. 2013. Impact of pesticide contamina-tion on aquatic microorganism populations in the littoralzone. *Arch Environ Contam Toxicol*, **64**: 399–409.

Li JQ, Shen J, Zhao XL and Hou Y. 2010. Isolation and characterization of 3,5,6-trichloro-2-pyridinol-degrading *Ralstonia* sp. strain T6. *Bioresource Technol*, **101**: 7479–7483.

Lipika M, Madhavi V, Mugdha H. 2014. Effect of UV and nitrous acid treatment on production of xylanase enzyme by *Acinetobacter* sp. *Inter J Current Microbiol Appl Sci*.,, **3**: 45-53.

Liu SS, Wang CL, Zhang J, Zhu XW and Li WY. 2013. Combined toxicity of Pesticide mixtures on green algae and photobacteria. *Ecotoxicol Environ Saf*, **95**: 98–103.

Malhat F and Nasr I. 2011. Organophosphorus pesticides residues in fish samples from the river Nile tributaries in Egypt. *Bull Environ Contam Toxicol*, **87**: 689–692.

Mallick BK, Banerji A, Shakil NA and Sethunathan NN. 1999. Bacterial degradation of chlorpyrifos in pure culture and in soil. *Bull Environ Contamination Toxicol.*, **62**: 48–55.

Maya KRS, Singh SN, Upadhyay SK and Dubey M. 2011. Kinetic analysis reveals bacterial efficacy for biodegradation of chlorpyrifos and its hydrolyzing metabolite TCP. *Process Biochem*, **46** : 2130–2136.

Metwally IMG. 2014. Bioremediation of water contaminated with pesticides. PhD dissertation, Al-Azhar University, Cairo, Egypt.

Monroe WS. 2005. Chapter 12: Molecular phylogenies and evolution, part three: The organic framework. Evolution.

Mulbry WW, Kearney PC, Nelson JO and Karns JS. 1987. Physical comparison of parathion hydrolase plasmids from *Pseudomonas diminuta* and *Favobacterium* sp. *Plasmid*, **18**: 173–177.

Muller HJ. 1930. Types of cisible variation induced by X-rays in *Drosophilia*. *J Gen*, **22**: 299-334.

Munazzah M, Khalil UR, Rahman AJ, Muhammad A, Ibraheem R, Sadia J and Nazish J. 2012. *Bacillus subtilis* improvement through UV and chemical mutagenesis for indigenously hyper produced Urate oxidase. *Pakistan J Life Social Sci.*, **10**:123-129.

Narasimhan A, Suresh S, Bist D and Shivakumar S. 2013. Enhancement of mycolytic activity of an antagonistic *Bacillus subtilis* through ethylmethane sulfonate (EMS) mutagenesis. *Turkish J Biol*, **37**: 323-328.

Nelson LM. 1982. Biologically-induced hydrolysis of parathion in soil. Isolation of hydrolyzing bacteria. *Soil Biol Biochem*, **14**: 219-222.

Racke KD, Laskowski DA and Schultz MR. 1990. Resistance of chlorpyrifos to enhanced biodegradation in soil. *J Agric Food Chem*, **38**: 1430–1436.

Raushel FM. 2002. Bacterial detoxification of organophosphate nerve agents. *Curr Opinion Microbiol*, **5**: 288–295.

Rowland RT. 1984. Industrial strain development: mutagenesis and random screening procedures. *Enzymes Microbial Technol.*, **6**: 3-11.

Salman A, Fard AT, Nasir A and Bokhari H. 2010. Comparative analysis of organophosphate degrading enzymes from diverse species . *Bioinfor.*, **5**: 67-72.

Sambrook J and Russell DW. 2001. **Molecular Cloning, A Laboratory Manual**, 3New York rdedition. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Serdar CM, Gibson DT, Munnecke DM and Lancaster HM. 1982. Plasmid involvement in parathion hydrolysis by *Pseudomonas diminuta*. *Appl Environ Microbiol.*, **44**: 246–249.

Sethunathan NN and Yoshida T. 1973. A Flavobacterium that degrades diazinon and parathion. *Can J Microbiol.*, **19**: 873–875.

Singh BK and Walker A. 2006. Microbial degradation of organophosphorus compounds. *FEMS Micrbiol Rev*, **30**: 428-471.

Singh BK, Walker A, Morgan JA and Wright DJ. 2004. Biodegradation of chlorpyrifos by *Enterobacter* strain B-14 and its use in bioremediation of contaminated soils. *Appl Environ Microbiol*, **70**: 4855–4863.

Thabit TMAM and El-Naggar MAH. 2013. Diazinon decomposition by soil bacteria and identification of degradation products by GC-MS. *Soil Environ*, **32**: 96-102.

Verma A, Dhiman K and Shirkot P. 2016. Hyper-production of laccase by *Pseudomonas putida* LUA15.1 through mutagenesis. *J Microbiol Exp*, **3**: 2-8.

Vijayabaskar P, Shankar RM and Sivakumar T. 2014. Effect of UV irradiation and ethylmethane sulphate on carboxy methyl cellulase production by *Bacillus pumilus*. *Int J Med Chem Anal*, **4**: 35-40.

Wang Y, Cang T, Zhao X, Yu R, Chen L,Wu C and Wang Q. 2012. Comparative acute toxicity of twenty-four insecticides to earthworm, *Eiseniaf etida*. *Ecotoxicol Environ Saf*, **79**: 122–128.

Wu F, Chen-Goodspeed M, Sogorb MA and Raushel FM. 2001. Enhancement, relaxation, and reversal of the stereoselectivity for phosphotriesterase by rational evolution of active site residues. *Biochem.*, **40**: 1332–1339.

Zheng Y, Long L, Fan Y, Gan J, Fang J and Jin W. 2013. A review on the detoxification of organophosphorus compounds by microorganisms. *Afr J Microbiol Res*, **7**: 2127-2134.

Zhongli C, Shunpeng L and Guoping F. 2001. Isolation of methyl parathion-degrading strain M6 and cloning of the methyl parathion hydrolase gene. *Appl Environ Microbiol*, **67**: 4922–4925.

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Molecular Clarifications of Grapevine Identities in Algerian Germplasm Collections using Microsatellite Markers

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Abstract

A total of twenty-three accessions of autochthonous grapevine cultivars from the collection of 'Tegnennif' Mascara in Northwestern Algeria were genotyped using nine nuclear microsatellite loci (SSR) to characterize their genetic diversity, and compare them with previous reports on cultivars at the germplasm collection of Skikda in Northeastern Algeria. The total number of alleles was sixty-three, the mean number of alleles per locus was 7.9, while the expected and observed heterozygosity values were 0.790 and 0.861 respectively. The most informative loci was VVMD5 with an effective number of alleles (Ne=6.62), while the low cumulative PI value was estimated to be 4.52×10^{-10} reflecting the high discriminative power of the chosen markers for the investigated set of grapevines. This study identifies three cases of synonyms within the collection, ten cases of real duplication, and four cases of homonymous grape cultivars. These involved nine different genotypes, six cases of pairs with the same name in both collections, while the remaining two cases are slightly different. In any case, the genetic relationship, based on the shared alleles' distance between the cultivars in both collections, showed very high levels of similarity between homonymous accessions in both collections which indicates a significant number of shared alleles in the studied loci. The results reported here are significant towards a better characterization of grapevine accessions and can help in future germplasm management and the breeding efforts in Algeria.

Keywords: Germplasm collection, SSR, Characterization, Genetic diversity, Homonyms, Synonyms.

1. Introduction

Among the processes necessary for the conservation of genetic resources is the establishment of genetic collections to build the first barrier against genetic erosion. Therefore, there is a big need to identity and clarify the real number, evaluate genetic diversity, and study relationships among accessions located in the smallest possible area. Germplasm collections of grapevines were created throughout the world with the aim to conserve the existing autochthonous diversity (El Oualkadi *et al*., 2009). The native genotypes are very well-adapted to local environmental conditions, and probably contain genes of different interests that could be considered as important resources to plant breeders and geneticists (Santana *et al*., 2008). One of the important troubles in grapevine cultivar identifications is the abundance of synonyms and homonyms at international, national, and regional levels, complicated by errors in the material propagation, altogether leading to a high number of mistakes and repetitions in germplasm banks (Sefc *et al*., 2000; Diaz-losada *et al*., 2013). During the last few years, accessions from germplasm collection of Skikda have already been identified and characterized using molecular analyses with nuclear and chloroplastic microsatellites (Laiadi *et al*., 2009) with ampelometric and SNP analyses (Zinelabidine *et al*., 2014). On the contrary, the accessions from the germplasm collection of Mascara have only been characterized through phyllometric measurements (Laiadi *et al*., 2013). However, the common morphological identification has several limitations, and does not necessarily provide enough evidence for the correct identification of the accessions (Nebish *et al*., 2017). Molecular markers such as SSRs (simple sequence repeats) or microsatellites have been demonstrated to be a powerful tool for cultivar identification, and are extensively used for the characterization of grapevine collections (Ibañez *et al*., 2003; Martin *et al*., 2003; This *et al*., 2004; Costantini *et al*., 2005; Santana *et al.*, 2008; Laiadi *et al.* 2009; Zinelabidine *et al*., 2014; Maletić *et al*., 2015), and for the verification of synonymies or homonymies (Sefc *et al*., 2000; Crespan and Milani, 2001; Schneider *et al*., 2001; Regner *et al*. 2006; Ferreira *et al*., 2015; Goryslavets *et al*., 2015; Nebish *et al*., 2017).

This study is conducted to evaluate the genetic diversity within the Mascara germplasm collection, and to identify duplicates, synonyms, homonyms, and genetic relationships of accessions after comparing them with previous reports of genetic profiles from the germplasm collection of Skikda that was considered a duplicate of the first one. This study aims at enhancing the understanding of genetic diversity among different genotypes in the germeplam bank of grapevines at the ITAFV 'Institut

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Technique de l'Arboriculture Fruitière et de la Vigne (ITAFV)' in Algeria.

2. Material and Methods

2.1. Plant Material and DNA Extraction

The plant materials consisted of twenty-three samples that were obtained from the grapevine collection located at the Experimental Station of Teghennif (Mascara) in Northwestern Algeria. These accessions belong to varieties that are classified as critically-endangered according to the 'Institut Technique de l'Arboriculture Fruitière et de la Vigne (ITAFV)' in Algeria.

Genomic DNA was isolated from young frozen leaves using the DNeasy^{TM} Plant Mini Kit (Qiagen, CA, USA). The DNA quality was determined on agarose gels (1 %) and the concentration was measured by NanoDrop ND-1000 spectrophotometer (Peqlab, Erlangen, Germany). Based on several criteria, the following nine microsatellites were selected and amplified in a multiplex PCR (Ibáñez *et al.,* 2009b): VVS2 (Thomas and Scott, 1993), VVMD5, VVMD27, VVMD28 (Bowers *et al.*, 1999), ssrVrZAG29, ssrVrZAG62, ssrVrZAG67, ssrVrZAG83 and ssrVrZAG112 (Sefc *et al*., 1999). All forward primers were labeled with either 6-FAM, VIC and NED. Labels and multiplex PCR amplifications were done according to Ibañez *et al.* (2009a) in a total volume of 25 µL containing 5 ng of genomic DNA.

2.2. Data Analysis

To detect identical genotypes, « Identity 1.0 » software (Wagner and Sefc, 1999) was used. The obtained profiles were compared with those published genotypes in the Skikda collection in Algeria (Laiadi *et al*., 2009). Several genetic diversity parameters were calculated using GenAlEx 6.41 (Peakall and Smouse, 2006): the number of alleles per locus (Na), the number of effective alleles (Ne), Shannon's Information Index, gene diversity or expected heterozygosity (He), probability of identity per locus (PI), and cumulative PI. The genetic distances between individual accessions were calculated as the allele-sharing distance (DAS) (Jin and Chakraborty, 1994), and a dendrogram based on the distance matrix was constructed using the neighbour-joining method (Saitou and Nei, 1987) by Populations v. 1.2.30 (http://bioinformatics.org, Langella, unpubl.), while Mega5.2 (Tamura *et al.,* 2011) was used to display it.

3. Results

3.1. Statistical Analyses.

A total of nineteen different SSR profiles were obtained for the twenty-three accessions studied with the nine markers (Table 1). Marker ssrVrZAG29 (ZAG29) showed a very low polymorphism in the present study: only two alleles and two genotypes in the whole population, and thus the polymorphism analysis was not used for the diversity study (Table 2). A total of sixty-three alleles were detected at the remaining eight SSR loci, ranging from 4 (ZAG83) to10 (VVS2) and 11 (VVMD28) and with an average of 7.87 per locus.

The most informative locus was VVMD5 with 6.62 effective alleles and the lowest probability of identity (PI= 0.041), followed by VVS2 (Ne=6.17; PI=0.045), while the least informative (apart from ZAG29) was ZAG83 with four alleles (Ne=3.02) and the highest probability of identical genotypes (PI=0.17). These findings are in line with results found by Boz *et al.* (2011) on fifty-five grape cultivars from Southeast Anatolia (Turkey) using fourteen SSR markers.

The observed heterozygosities (Ho) were very high, with a mean value of 0.861. The highest level (0.94) was detected at VVMD27 and ZAG 67, while the lowest (0.68) was at ZAG83.

Values of Probability of Identity (PI) ranged between 0.041 and 0.168, with four out of eight markers close to the value of 0.050 at which a grapevine microsatellite is considered hyper- polymorphic (Sefc *et al.,* 2001).The cumulative PI or probability to obtain individuals with identical profile at all eight loci was estimated as 4.52 X 10^{-10}, similar to those found by other authors for the same number of markers in grapevine, such as 6.93 X 10^{-12} (Ibáñez *et al.,* 2003) or 1.2 X 10^{-8} (Hvarleva *et al.,* 2004).

Table 1. Genetic profiles of 23 Algerian *V. vinifera* L. cultivars analyzed at 9 microsatellite loci. Allele sizes are given in base pairs (bp)

N°	Geno-type	Cultivar	VVS2		VVMD5		VVMD27		VVMD28		ZAG29		ZAG62		ZAG67		ZAG83		ZAG112	
1	**1**	Aberkane	137	137	236	240	183	194	239	263	109	109	196	200	137	153	190	192	236	236
2	**2**	Aneb el Cadi	143	145	232	236	189	194	249	263	109	109	188	200	137	137	195	195	232	238
3	**3**	Adadi des Bibans	133	137	232	240	183	189	247	261	109	109	186	204	123	137	190	195	227	232
4	**3**	Aïn el Couma	133	137	232	240	183	189	247	261	109	109	186	204	123	137	190	195	227	232
5	**4**	Bouaber des Aures	133	143	232	232	191	194	247	261	109	109	204	204	123	129	190	190	236	240
6	**3**	Ahchichene	133	137	232	240	183	189	247	261	109	109	186	204	123	137	190	195	227	232
7	**5**	Aïn el Kelb	135	143	236	240	181	189	247	261	109	109	188	204	129	137	195	195	227	238
8	**6**	Ahmar Mechtras	135	147	232	238	183	194	251	257	109	109	192	204	137	153	195	201	232	238
9	**7**	Valenci Noir	133	135	240	242	194	194	237	263	109	109	194	204	129	137	192	195	227	229
10	**1**	Muscat Noir	137	137	236	240	183	194	239	263	109	109	196	200	137	153	190	192	236	236
11	**8**	Torki	133	135	240	242	//	//	235	261	109	109	194	204	123	137	195	195	227	229
12	**9**	Tizi Ounine1	133	143	226	240	183	185	247	261	109	111	188	200	129	137	192	195	227	232
13	**10**	Ghanez	133	137	236	238	183	194	247	251	109	109	200	204	129	137	190	201	236	238
14	**11**	Elwali	137	143	240	242	183	194	261	261	109	109	200	204	123	137	190	195	227	236
15	**12**	Farrana	143	145	228	240	179	194	251	261	109	109	186	186	147	149	195	195	227	227
16	**13**	Bezzoul el Khadem	133	143	238	238	179	181	247	261	109	111	188	188	129	158	190	190	229	229
17	**14**	Tizi Ounine 2	145	151	226	228	181	185	261	263	109	111	188	204	137	158	192	195	232	236
18	**15**	SbaaTolba	151	153	238	242	183	189	260	263	109	109	188	188	//	//	195	201	227	234
19	**7**	Tadelith	133	135	240	242	194	194	237	263	109	109	194	204	129	137	192	195	227	229
20	**16**	Sidi Ahmed draa el Mizen	137	155	226	242	179	194	247	261	109	109	188	204	123	137	190	192	227	240
21	**17**	Raisin de Bouni	137	149	226	248	185	194	237	247	109	109	188	204	123	137	190	192	232	245
22	**18**	El mokrani	133	149	232	240	179	194	237	247	109	111	192	204	137	153	190	195	227	245
23	**19**	Muscat d'Adda	133	133	226	228	179	185	271	271	109	109	192	204	123	147	190	195	232	259

Table 2. Genetic parameters obtained in eight SSR markers for 19 distinct genotypes: Statistical results for 8 microsatellite markers used in the present study, namely Observed number of alleles (Na), effective number of alleles (Ne), Shannon's Information index (I), observed heterozygosity (Ho), expected heterozygosity (He), and Probability of identity.

Locus	Na	Ne	I	Ho	He	PI
VVS2	10	6.171	2.009	0.895	0.838	0.045
VVMD5	8	6.624	1.966	0.895	0.849	0.041
VVMD27	7	4.985	1.754	0.944	0.799	0.066
VVMD28	11	5.685	2.001	0.895	0.824	0.051
ZAG62	7	4.198	1.638	0.789	0.762	0.090
ZAG67	7	3.951	1.610	0.944	0.747	0.096
ZAG83	4	3.021	1.216	0.684	0.669	0.168
ZAG112	9	5.823	1.937	0.842	0.828	0.050
Sum	63	40.457				
Mean	7.9	5.057		0.861	0.790	
Cumulative						4.52×10^{-10}

3.2. Identification of Synonyms

nSSR markers identified nineteen out of the twenty-three genotypes analyzed, (Table 1). Given the low value found for cumulative PI, cultivar names with identical genotypes could be considered as synonyms, if no critical morphological differences are found between them such as berry color.

Ahchichene had been previously shown to be a synonym for Adari des Bibans in the collection of Skikda (Laiadi *et al.,* 2009; Zinelabidine *et al.,* 2014). In this work, this synonym has been confirmed, and Aïn el Couma also showed the same DNA profile, while it had been reported by the same authors as a different cultivar in Skikda collection. Muscat Noir matched Aberkane, which means 'black' in the Berber dialect in Northern Algeria; it refers to the berry colour. Tadelith and Valenci Noir, both with a black berry color, also had the same DNA profiles.

3.3. Cluster Analysis

Considering the twenty-three accessions analyzed here as well as the twenty-seven unique accessions from Skikda collection (Laiadi *et al*., 2009), thirty-six non-redundant cultivars were found from both collections, with five common SSR markers in both studies. A dendrogram based on a genetic distance measure from those markers was constructed using the Weighted Neighbour-Joining method for a preliminary evaluation of genetic relatedness between the investigated cultivars (Figure 1). Accessions could be easily distinguished in the dendrogram despite being homonymous. When five similar pairs were shared, there is a significant number of alleles for the five loci 'Bouni'or 'raisin de Bouni'(≠ 2 /10 alleles) , 'Tizi Ouinine (S) with Tizi Ouinine2 (M) (≠ 4 /10 alleles) and 'Ghanez' (≠ 4 /10 alleles). The last pair is considered as the unique pair in a separate group that did not appear close to other accessions. 'Tadelith', 'SbaaTolba', 'Elmokrani' or 'Amokrane' in the Skikda collection are three cases of homonymous pairs. They were organized in different sub clusters which in fact did not show a clear relationship between them for the five loci used in this comparison.

As for 'Elmokrani' and 'Elwali' from Mascara , at first they were thought to be replicated from 'Amokrane' or its synonyms 'Louali' from Skikda, but eventually it seems to be completely different between them and among other groups, as well as between those cultivated in the Skikda collection. The same cases were recorded with 'Raisin de Bouni' in Mascara and 'Bouni' in Skikda collection, where it was thought that the name used for the same cultivar can be changed in different growing areas, but it turned out that they were different at least in two alleles with both loci VVMD5 and VVMD28. Finally 'Farana de Mascara' or 'Farrana' was different from that called 'Farrana Noir' regarding the color of the berry which was black in the latter and white in the former although they were located in the same branch in the cluster.

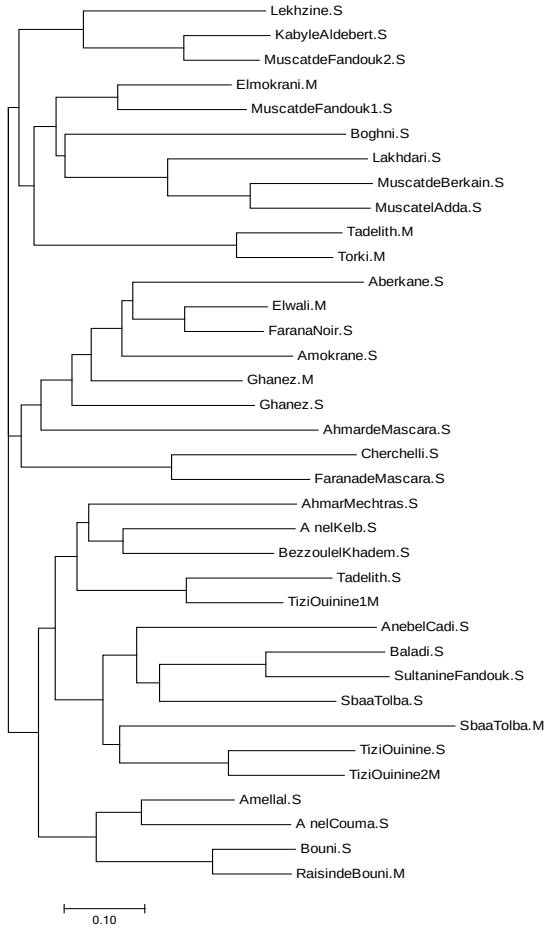

Figure 1. Dendrogram of 36 Algerian grapevine cultivars genotyped at five nSSR loci. The dendrogram was built using the Weighted Neighbour-Joining method with Populations software 1.2.32. (M. Mascara, S. Skikda).

4. Discussion

4.1. SSR Genetic Diversity

The results obtained from statistical analyses agree well with those of previous works on Bulgarian (Hvarleva, 2004) and Turkish genotypes (Boz *et al.,* 2011), in particular in the variation range of alleles per locus; VVMD28 was also found among the most discriminating loci in a group of Muscats by Crespan and Milani (2001).

The values of observed heterozygosity are similar to those found by some previous studies on grapevines, such as 84.3 % in Castilian cultivars obtained with the GENRES 081 set of six nuclear loci (Santana *et al.,* 2008) and higher than 81.0 % for ninety-six genotypes (Ibáñez *et al.* 2003), 80.6 % for 163 genotypes (Martín *et al.,* 2003), and 78.4 % for seventy-three genotypes (Fernández-González *et al.,* 2007). These high values of heterozygosity may indicate that most grapevine cultivars were originally obtained through hybridization, and fixed later by clonal propagation. The gene diversity or expected heterozygosity (He) values were lower than the Ho values for every marker, which may indicate either the absence of null alleles or to their very low frequency (Ibáñez *et al.,* 2003). Gene diversity varied between 0.67 for ZAG83 and about 0.85 for VVS2, VVMD5, while the average gene diversity value was 0.79, similar to that found by other authors for He (Díaz-Losada *et al.,* 2013; Goryslavets *et al.,* 2015)

4.2. Management of Germplasm Collections

The comparison of the nineteen non-redundant genotypes of Mascara accessions with the twenty-seven previously published for the Skikda collection (Laiadi *et al.,* 2009) showed some matches. Many accessions conserved in Mascara are considered as duplicated in the Skikda collection. Several cases were detected in Table 3: Aberkane (1 in Table 1), Aneb el Cadi (2), Aïn el Kelb (5), Bezzoul el Khadem (16), and Muscat d'Adda (23), show the same names and genotypes in both collections; Ahmar Mechtras from Mascara and Ahmar de Mascara from Skikda are two cultivars with the same first name 'Ahmar' meaning red in Arabic; this cultivar from Mascara is different in the second name only. Ahchichene (6) and its synonym Adadi des Bibans (3) from the Mascara collection also matched in names and genotypes in both collections with an additional synonym, Lekhzine, in the Skikda collection as reported by Laiadi *et al.* (2009). The synonym of Sidi Ahmed Draa el Mizen (20) in the Mascara collection occurred as a result of changes in the grammatical gender of the denomination (Díaz-Losada *et al.,* 2013) with Ahmed Draa el Mizen presented by Amellal in Skikda collection. Each of these pairs shares the same berry color. 'Farrana' (15) or 'Farhana' meaning 'happy' in Arabic according to Levadoux *et al.* (1971) shows the same genotype with Farana de Mascara.

Table 3. Genotypic identities of Mascara and Skikda germoplasm collections.

Accessions name of Skikda	Identical genotypes from Mascara	Berry colour
Lekhzine.S	Adadi des Bibans.M	White
Aberkane S	Aberkane.M	Black
Amellal.S	Sidi Ahmed Draa el Mizen.M	White
Aneb el Cadi.S	Aneb el Cadi.M	White
Muscat d'Adda.S	Muscat el Adda.M	Black
Aïn el Kelb.S	Aïn el Kelb.M	White
Bezzoul el Khadem.S	Bezzoul el Khadem.M	Black
Ahmar de Mascara.S	Ahmar Mechtras.M	Pink to Red
Farrana de Mascara.S	Farrana.M	White
Kabyle Aldebert.S	Bouaber des Aures.M	Black

Four groups of homonyms were detected between the two collections involving nine different genotypes (Table

4): cases of pairs among them taking the same names in both collections are 'Ghanez' 'SbaaTolba' 'Tadelith' and 'Tizi Ouinine 1, 2'.

Form the comparison with previous results by the same authors regarding the Skikda germplasm collection published in 2009 and 2014, nine synonyms affecting eighteen genotypes were detected. False duplicated or homonyms were found for four cultivars, involving ten genotypes. Nine genotypes were identified in the collection of Mascara that have not been described previously using SSRs.

Thus, the total current number of accessions to represent local grapevines maintained at the both collections is thirty-six.

Table 4. Homonymous grapevine cultivars analyzed at 5 microsatellite loci (allele sizes are given as base pairs). S. accession from Skikda collection (Laiadi *et al*. 2009). M. accession from Mascara collection.

N°	Homonymous Cultivar name	VVS2		VVMD5		VVMD27		VVMD28		VrZAG62	
1	Ghanez (S)*	143	145	228	240	183	194	247	251	200	204
	Ghanez (M)	133	137	236	238	183	194	247	251	200	204
2	SbaaTolba (S)*	133	145	226	234	179	194	221	255	188	204
	SbaaTolba(M)	151	153	238	242	183	189	260	263	188	188
3	Tadelith (S)*	133	143	226	226	179	183	251	261	188	200
	Tadelith (M)	133	135	240	242	194	194	237	263	194	204
4	TiziOuinine (S)*	145	151	226	228	185	194	261	261	188	188
	TiziOuinine 2 (M)	145	151	226	228	181	185	261	263	188	204
	TiziOuinine 1 (M)	133	143	226	240	183	185	247	261	188	200

5. Conclusion

The present study provides the first molecular database for Algerian grapevine germplasm management. The germplasm collection of 'Teghennif' thus contains nineteen out of the twenty-three analyzed accessions of autochthonous original cultivars with three cases of recorded duplications involving seven genotypes. This study shows the important genetic diversity in germplasm collections for *Vitis* in Algeria. Unfortunately, only five SSRs were utilized in the comparison of profiles; however it becomes clear now that during the transfer of samples there was a clear confusion in the proper labeling among the cutting materials that were transferred from the mother germplasm collection. In practice, stickers on cutting materials may be easily dropped completely, leading to a possible mislabeling especially if the person who transferred the cutting is not specialized in the field. The right thing to do in such cases is to remove all questionable material from the GenBank and replace it with true to type cutting material, thus, reducing the cost of effort, time, and money to verify its identity.

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Author contributions

All authors have participated in the research and article preparation. Rahali M. and Achour H. have participated in the analysis and interpretation of data and manuscript revision.

Conflict of interest disclosure

The authors declare no conflict of interest.

References

Bowers JE, Dangl GS and Meredith CP. 1999. Development and characterization of additional microsatellite DNA markers for grape. *Am J Enol Vitic*, **50**: 243-246.

Boz Y, Bakir M, Çelikkol BP, Kazan K, Yilmaz F, Çakir B. Aslantaş Ş. Söylemezoğlu G, Yaşasin AS, Özer C, Çelik H and Ergül A. 2011. Genetic characterization of grape .*Vitis vinifera* L.. germplasm from Southeast Anatolia by SSR markers. *Vitis*, **50**: 99–106.

Costantini L, Monaco A, Vouillamoz JF, Forlani M and Grando MS. 2005. Genetic relationship among local *Vitis vinifera* cultivars from Campania (Italy). *Vitis*, **44**: 25-34.

Crespan M and Milani N. 2001. The Muscats A molecular analysis of synonyms, homonyms and genetic relationship within a large family of grapevine cultivars. *Vitis*, **40**: 23-30.

Díaz-Losada E, Tato Salgado A, Orriols-Fernández I, Ramos-Cabrer AM and Pereira-Lorenzo S. 2013. New Synonyms and Homonyms for Cultivars from Northwestern Spain. *Am J Enol Vitic*, **64**: 156-162.

El Oualkadi A, Ater M, Messaoudi Z, Laucou V, Boursiquot JM, Lacombe T and This P. 2009. Molecular characterization of Moroccan grapevine germplasm using SSR markers for the establishment of a reference collection. *J Int Sci Vigne Vin*, **43**: 135-148.

Fernández-González M, Mena A, Izquierdo P and Martínez J. 2007. Genetic characterization of grapevine (*Vitis vinifera* L.) cultivars from Castilla La Mancha .Spain. using microsatellite markers. *Vitis*, **46**: 126-130.

Ferreira V, Pinto-Carnide O, Mota T, Martín JP, Ortiz JM and Castro I. 2015. Identification of minority grapevine cultivars from Vinhos Verdes Portuguese DOC Region . *Vitis*, **54** (Special Issue) 53–58.

Goryslavets S, Bacilieri R, Risovannaya V, Memetova E and Laucou V. 2015. Genetic diversity of ancient grape cultivars of the Crimea region. *Vitis*, **54** (Special Issue): 37-41.

Hvarleva T, Rusanov K, Lefort F, Tsvetkov I, Atanassov A and Atanassov I .2004. Genotyping of Bulgarian *Vitis vinifera* L. Cultivars by microsatellite analysis. *Vitis*, **43**: 27-34.

Ibáñez J, De Andres M T, Molino A and Borrego J. 2003. Genetic study of key spanish grapevine varieties using microsatellite analysis. *Am J Enol Vitic*, **54**: 22-30.

Ibáñez J, Vargas AM, Palancar M, Borrego J and De Andrés MT. 2009a. Genetic relationships among table-grape varieties. *Am J Enol Vitic*, **60**: 35-42.

Ibañez J, Velez MD, De Andres MT and Borrego J. 2009b. Molecular markers for establishing distinctness in vegetatively propagated crops a case study in grapevine. *Theor Appl Genet*, **119**:1213-1222.

Laiadi Z, Bentchikou MM, Bravo G, Cabello F and Martínez-Zapater J. 2009. Molecular identification and genetic relationships of Algerian grapevine cultivars maintained at the germplasm collection of Skikda (Algeria). *Vitis*, **48**: 25-32.

Laiadi Z, Bencharif S, Lakhrif Z, Bentchikou M M and Mohand-Larbi R. 2013. First ampelometric study of autochthonous grapevines in Algeria Germplasm collection of Mascara. *Vitis*, **52**: 21–27.

Levadoux L, Benabderrabou A and Douaouri B. 1971. Ampélographie Algérienne : Cépages de Cuve et de Table Cultivés en Algérie. SNED.

Maletić E, Pejić I, Karoglan Kontić J, Zdunić, Preiner D, Šimon S, Andabaka Ž, Žuljmihaljević M, Bubola M, Marković Z, Stupić D and Mucalo A. 2015. Ampelographic and genetic characterization of Croatian grapevine varieties. *Vitis*, **54** (Special Issue): 93–98.

Martin JP, Borrego J, Cabello F and Ortiz JM. 2003. Characterization of Spanish grapevine cultivar diversity using sequence-tagged microsatellite site markers. *Genome*, **46**: 10-18.

Nebish A, Ochssner I, Maul E, Topfer R, Hausmann L, Hovhannisyan A, Devejyan H, Melyan G and Aroutiounian R. 2017. Genetic identification and characterization of Armenian grapevine cultivars. 40th World Congress of Vine and Wine, BIO Web of Conferences 9, 01020 **.** *Sofia, Bulgaria.*

Peakall R and Smouse PE. 2006. GENALEX 6 genetic analysis in excel population genetic software for teaching and research. *Mol Ecol Notes*, **6**: 288-295.

Regner F, Hack R and Santiago JL. 2006. Highly variable Vitis microsatellite loci for the identification of Pinot Noir clones. *Vitis*, **45**: 85-91.

Saitou N and Nei M .1987. The neighbor-joining method - a new method for reconstructing phylogenetic trees. *Mol Biol Evol*, **4**: 406-425.

Santana JC, Hidalgo E, De Lucas AI, Recio P, Ortiz JM, Yuste J, Arranz C and Rubio JA . 2008. Identification and relationships of accessions grown in the grapevine (*Vitis vinifera* L.) germplasm bank of Castilla y Léon .Spain. and the varieties authorized in the VQPRD areas of the region by SSR-marker analysis. *Genet Resour Crop Evol*, 55: 573-583.

Schneider A, Carra A, Akkak A, This P, Laucou V and Botta R .2001. Verifying synonyms between grape cultivars from France and Northwestern Italy using molecular markers. *Vitis*, **40**: 197-203.

Sefc KM, Regner F, Turetschek E, Glossl J and Steinkellner H. 1999. Identification of microsatellite sequences in *Vitis riparia* and their applicability for genotyping of different *Vitis* species. *Genome*, **42**: 367-373.

Sefc KM, Lopes MS, Lefort F, Botta R, Roubelakis-Angelakis KA, Ibáñez J, Pejic I, Wagner HW, Glossl J and Steinkellner H. 2000. Microsatellite variability in grapevine cultivars from different European regions and evaluation of assignment testing to assess the geographic origin of cultivars. *Theor Appl Genet*, **100**: 498-505.

Sefc KM, Lefort F, Grando MS, Scott KD, Steinkellner H and Thomas, MR. 2001. Microsatellite markers for grapevine a state of the art. *In:* **Molecular Biology & Biotechnology of Grapevine,** Roubelakis-Angelakis KA.(Ed.). Kluwer Academic Publishers, The Netherlands .pp 30.

Tamura K, Peterson D, Peterson N, Stecher G, Nei M and Kumar S .2011. MEGA5 Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol Biol Evol*, **28**: 2731-2739.

This P, Jung A, Boccacci P, Borrego J, Botta R, Costantini L, Crespan M, Dangl GS, Eisenheld C, Ferreira-Monteiro F, Grando MS, Ibáñez J, Lacombe T, Laucou V, Magalhães M, Meredith CP, Milani N, Peterlunger E, Regner F, Zulini L and Maul E. 2004. Development of a standard set of microsatellite reference alleles for identification of grape cultivars. *Theor Appl Genet*, **109**: 1448-1458.

Wagner HW, Sefc KM .1999. Identity 1, Centre for Applied Genetics, University of Agricultural Science, Vienna.

Zinelabidine LH, Laiadi Z, Benmehaia R, Gago P, Boso S, Santiago JL, Haddioui A, Ibáñez J, Martínez-Zapater JM and Martínez MC. 2014. Comparative ampelographic and genetic analysis of grapevine cultivars from Algeria and Morocco. *Aust J Grape Wine R*, **20**: 324–333.

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A Comparative Study of Antibiotics and Probiotics against Pathogens Isolated from Coastal Shrimp Aquaculture System

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Abstract

Bacterial diseases are increasing at an alarming rate in the shrimp aquaculture production systems. To control microbial diseases, a number of antimicrobial agents including antibiotics are used in shrimp farms which led to problems such as antibiotic resistance. Therefore, the use of natural bacterial isolates or probiotics as an alternative method for the control of pathogenic bacterial strains is gaining popularity. In this study, seven shrimp pathogens were isolated from a coastal shrimp aquaculture system. Then, some common antibiotics and commercially available probiotics such as *Bacillus*. spp, *Pediococcus*. spp. were applied against the pathogens. For antibiotics, the disc diffusion method was used, whereas the well diffusion method was used for probiotics. Two common pathogens of shrimp hatcheries, namely *V. parahaemolyticus* and *V. vulnificus* showed resistance against antibiotic cephalosporin and streptomycin. On the other hand, both probiotic bacteria exhibited good results against all the pathogens including *V. parahaemolyticus* and *V. vulnificus* except probiotic *Bacillus* spp against *Bacillus fastidiosus.* These results demonstrated that the use of probiotic bacteria within the shrimp aquaculture could be a good solution for decreasing pathogenic microorganisms and reducing the antibiotic resistance problem in shrimp hatcheries.

Keywords: Antibiotic resistance, Probiotics, Shrimp pathogens.

1. Introduction

The aquaculture industry is considered as one of the major contributors to global food production. The growth of the aquaculture industry is hampered by unpredictable mortalities, many of which are caused by pathogenic microorganisms. Bacterial diseases have been attributed to biological production bottlenecks in intensive aquaculture, hence necessitating the use of chemicals such as drugs and antibiotics in health management strategies (Newaj-Fyzul *et al*., 2015). The application of antibiotics had been an effective strategy only at the beginning, but the residuals remaining in the rearing environment exert selective pressures for long periods of time, and this has become a big challenge for health management (Lakshmi *et al*., 2013). The indiscriminate use of antibiotics resulted in the emergence of antibiotic-resistant bacteria in aquaculture environments, the increase of antibiotic resistance in fish pathogens, transfer of these resistance determinants to the bacteria of land animals and to human pathogens, and in alterations of the bacterial flora both in sediments and in the water column (Verschuere *et al.*, 2000). An alternative method for controlling pathogenic bacterial strains in shrimp cultures could be the supplementation with pure cultures of natural bacterial isolates (biocontrol or use of probiotics) which might produce chemical substances inhibiting the growth of pathogens. The approach basically employs the activity of microorganism that could suppress or inhibit the growth of *V. harveyi*without causing a bad impact on the equilibrium system in a particular microbial community. (Ohira *et al.*, 1996). This research is an attempt to present a comparative study of the efficacy of conventional antibiotics and probiotics against some pathogens isolated from coastal shrimp aquaculture systems.

2. Materials and Methods

2.1. Sample Collection

Water, soil, raw water, treated water, and water from post-larva culture were collected from a total of seven shrimp hatcheries and grow-out ponds of Cox's Bazar, Bangladesh. The samples were taken in sterile containers, and were immediately transferred to the laboratory.

2.2. Enumeration and Isolation of Bacteria

A Nutrient agar medium and a Thiosulfate Citrate Bile Salts Sucrose (TCBS) agar medium were used for the enumeration of bacteria. Serial dilution up to 10^6, pour plate and spread plate (Sanders *et al*., 2012) methods were applied for the total count. The inoculated media were incubated at 37^0C for twenty-four to forty-eight hours. After incubation, the plates having well-spaced colonies

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were placed on a colony counter (Stuart Scientific U K). The colonies were counted and calculated by multiplying the average number of colonies per plate by reciprocal of the dilution factor. The calculated results were expressed as colony forming units (CFU) per mL of the sample. The colonies were selected for isolation on the basis of colony morphology including elevation, margin, and surface. The colonies were then transferred to nutrient agar slants and purified through the streak plate method. The pure cultures of the isolates were coded and kept in polythene bags and preserved as a stock culture in the refrigerator at 4^0C for further study.

2.3. Identification of Selected Isolates

The selected isolates were subjected to biochemical tests, and the results were compared with the standard descriptions given in "Bergey's Manual of Determinative Bacteriology", 8th ed. (Buchanon and Gibson 1974) and 9th ed. (Halt *et al*., 2000). The tests included Gram-staining, spore staining, acid-fast staining, starch hydrolysis, Voges Proskauer (V-P) test, production of H_2S, gelatin liquefaction, nitrate reduction, indole, deep glucose agar, catalase reaction, methyl-red, carbohydrate fermentation, urease , motility , oxidase. Cultural and physiological studies were also done.

2.4. Antibiotic Susceptibility Test (Bauer et al. 1966)

The isolates were subjected to the discs diffusion method for antibiotic susceptibility against common antibiotics. The test was performed on Mueller Hinton agar plates. The suspension of the isolates was prepared using sterile distilled water, and was adjusted to 0.5 McFarland standards. A 100µL suspension of freshly-grown bacterial cultures was spread on Mueller Hinton agar plates. The antibiotic discs were placed on the surface of the agar and kept at 4^0C for thirty minutes. Then, the plates were incubated at 37ºC for twenty-four to forty-eight hours. Chloramphenicol (30µg), Penicillin G (10 Units), Erythromycin (15µg), Nitrofuran (30 µg), Rifampicin (5µg), Cephalosporin (30 µg), and Streptomycin (10µg) (Manufacturer: Oxoid) were used to observe the susceptibility pattern of the isolates.

2.5. Probiotic Efficacy Test (Vijayan et al. 2006)

Overnight culture filtrates of two probiotic bacteria *Bacillus* spp. and *Pediococcus* spp (Manufacturer: Lactospore) were used in the well diffusion method (Magaldi *et al*. 2004) for the probiotic efficacy test. The selected isolates were heavily seeded in the nutrient agar plate. Then a hole was made in media by a sterile cork borer in aseptic condition, and one drop of the malted agar was poured into the hole to make a base layer. 0.1 mL culture filtrates of probiotic bacteria (*Bacillus* spp. and *Pediococcus* spp.) were poured into two separate holes. The culture plates were kept at a low temperature (4ºC) for two-four hours for a maximum diffusion. The plates were then incubated at 37ºC for twenty-four hours. The efficacy of the probiotic was determined by measuring the zone of inhibition expressed by the diameter in millimeter. The experiment was carried out more than once, and the mean of reading was taken.

3. Results

3.1. Enumeration of Total Count:

The total bacterial count and *Vibrio* load count of the collected samples are shown in Table 1. There is a variation in the bacterial count and *Vibrio* load count among different types of samples on the Nutrient agar medium and TCBS agar medium (Figure 1)

Figure 1. *Vibrio* Load Count on TCBS Agar Medium

Table 1. Total bacterial count and *Vibrio* load count of the collected samples at selected sampling sites.

Sl. No.	Location	Type of Sample	Total Bacterial Count (CFU/mL)	*Vibrio* Load Count (CFU/mL)
1.	Mixing water zone at Kolatali, Cox's Bazar	Water sample	2.25×10^3	2.17×10^2
		Soil sample	6.24×10^3	4.14×10^3
2.	Pioneer Shrimp Hatchery Limited	Raw water	3.19×10^4	2.01×10^4
		Treated water	2.31×10^2	3.76×10^3
3.	Golden Shrimp Hatchery Limited	Water sample	2.56×10^4	3.22×10^4
		Soil sample	3.18×10^4	4.54×10^4
4.	Mixing water zone at Sonapara, Cox'sBazar	Water sample	5.54×10^2	2.91×10^2
		Soil sample	2.37×10^2	4.55×10^3
5.	United Hatchery Limited, Cox's Bazar	Raw water	2.37×10^3	2.55×10^3
		Treated water	4.6×10^2	2.32×10^2
		Water from algal culture	2.09×10^2	4.61×10^2
6.	Modern Hatchery Limited, Cox's Bazar	Raw water	3.18×10^3	3.54×10^3
		Treated water	2.15×10^2	0
		Water from post larval culture	2.03×10^3	3.29×10^3
7.	Baley Shrimp Hatchery	Raw water	4.31×10^3	2.43×10^3

3.2. Identification of Selected Isolates

During the period of the study, a total of twenty bacterial colonies were isolated according to their morphological characteristics. Seven isolates (Coded as AM1 to AM7) were finally selected from seven groups for a detailed examination. The bacterial isolates were

characterized according to their morphological characteristics including the size and shape of the organism, the arrangement of the cells, presence or absence of the spores, regular or irregular forms, gram reaction etc. The cultural and physiological characteristics include temperature tolerance, salt tolerance, IMViC test, H_2S production, nitrate reduction, deep glucose agar test, fermentation of different carbohydrates etc. (Table 2). All these characteristics were then compared with the standard descriptions of ''Bergey'sManual of Determinative Bacteriology'', 8th ed. (Buchanon and Gibson 1974) and were found to be closely-related to the species given below. (Table 3**)**

Table 2. Morphological and biochemical test results of selected isolates.

Parameters	AM1	AM2	AM3	AM4	AM5	AM6	AM7
Vegetative cells	Short rod (0.3-1.0 µm	Curved rod (0.5-0.8 µm	Short rod (1.75-2.63µm	Curved rod (0.5-0.8 µm	Straight rod (1.1-1.5 µm	Curved rod (1.1-1.5 µm	Straight rod (0.5-0.8 µm
Cell arrangement	Single or in pair	Single	Single, pair, short chain.	Single	Single or in pair	Single	Single or in pair
Gram staining	Gram -ve	Gram -ve	Gram +ve	Gram -ve	Gram -ve	Gram –ve	Gram -ve
Spore staining	Non-spore former	Non-spore former	Spore former	Non-spore former	Non-spore former	Non-spore former	Non-spore former
Motility test	Motile	Motile	Non motile	Motile	Motile	Motile	Motile
Catalase test	+	+	+	+	+	-	-
Glucose broth	Turbid growth	Turbid growth	Turbid growth	Turbid growth	Turbid growth	Turbid growth	Turbid growth
Deep glucose agar test	Facultative Anaerobic	Facultative Anaerobic	Aerobic	Facultative Anaerobic	Facultative Anaerobic	Facultative Anaerobic	Facultative Anaerobic
Casein hydrolysis	-	+	-	+	+	+	-
Starch hydrolysis	-	+	+	+	+	+	-
Egg albumin test	-	-	+	-	+	+	-
Gelatin liquefaction	+	+	-	+	-	+	+
Growth in synthetic media	-	-	-	-	-	-	-
Growth in inorganic salt	+	+	-	+	-	+	+
Citrate utilization	-	+	-	+	-	+	Variable
Voges-Proskauer test	-	-	-	-	-	-	Variable
Methyl red test	+	+	-	+	+	+	+
Nitrate reduction test	+	+	+	+	+	+	+
H2S production	-	-	+	-	-	-	-
Indole test	-	Variable	-	+	+	+	+
Urease test	-	-	+	-	-	+	-
Oxidase test	+	+	-	+	-	+	+
glucose,	Acid and gas	+	No acid and gas	Acid without gas	Acid from	Acid but no gas	Acid and gas
Fructose	Acid and gas	+	Acid without gas	Acid and gas	Acid from	Alkali without gas	Alkali without gas
Galactose	Acid and gas	+	Alkali without gas	Alkali without gas	Acid from	Acid and gas	Acid and gas
Sucrose	Alkali without gas	-	No acid and gas	Acid and gas	Alkali without gas	Acid and gas	Acid but no gas
Lactose	Alkali without gas	-	Alkali without gas	Acid without gas	Acid from	Alkali without gas	Acid and gas
Xylose,	Alkali without gas	-	No acid and gas	Alkali without gas	Alkali without gas	Alkali without gas	Alkali without gas
Arabinose	Alkali without gas	+	No acid and gas	Acid and gas	Acid from	Alkali without gas	Acid but no gas
Maltose	Alkali without gas	+	No acid and gas	Acid without gas	Alkali without gas	Acid but no gas	Acid but no gas
Mannitol	Alkali without gas	+	Alkali without gas	Acid without gas	Acid from	Acid and gas	Acid but no gas
pH 4.5	++++	++	-	-	+++	+	+
pH 6.5	++++	+++	++++	+++	+++	++	++
pH 7.5	+++	+++	+++	+++	+++	+++	+++
pH 8.5	+++	++	-	-	++	++	+++
Temperature (5 ^{0}C)	-	-	+	+	-	-	-
Temperature (10 ^{0}C)	-	+	+	+	-	-	-
Temperature (27 ^{0}C)	+++	+++	+++	+++	+++	+++	+++
Temperature (37 ^{0}C)	+++	+++	+++	+++	+++	+++	+++
Temperature (45 ^{0}C)	-	-	-	-	-	-	-

Note: Positive (+ =Scanty, ++ = Moderate, +++ = Heavy), - = Negative

Table 3. Species Name of Selected Isolates.

Code of Isolates	Name of Species
AM1	*Aeromonas salmonicida*
AM2	*Vibrio parahaemolyticus*
AM3	*Bacillus fastidiosus*
AM4	*Vibrio vulnificus*
AM5	*Escherichia. Coli*
AM6	*Vibrio harveyi*
AM7	*Aeromonas bestiarum*

3.3. Antibiotic Susceptibility Test and Probiotic Efficacy Test

The antibiotic susceptibility test of the selected isolates was performed by the disc diffusion method using the standard discs (Figure 2). The probiotic efficacy tests (Figure 3) were done by the well diffusion method. Figure 4 presents comparative results of antibiotic susceptibility and probiotic efficacy against the identified pathogens.

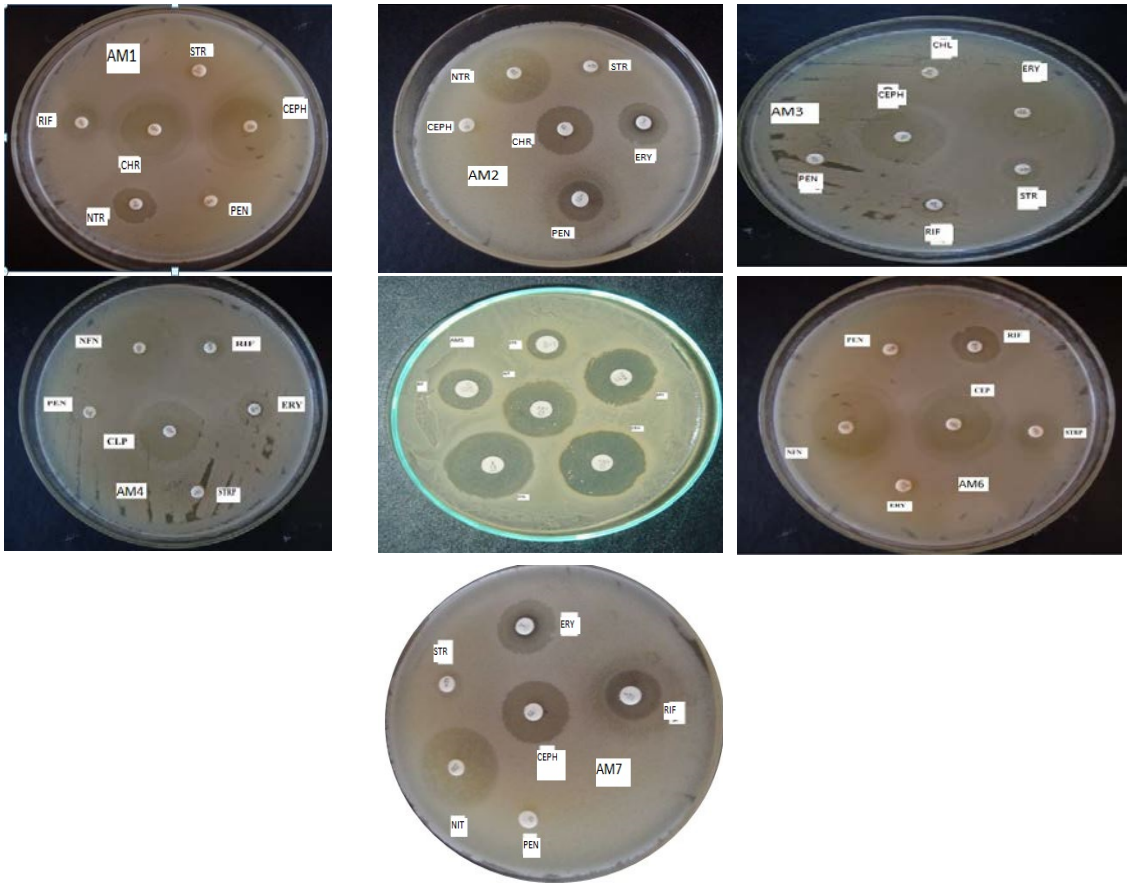

Figure 2. Antibiotic Susceptibility of isolates AM1 to AM7

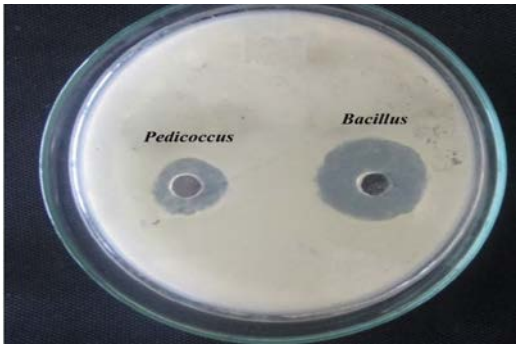

Figure 3. Probiotic efficacy of *Bacillus* and *Pedicococcus* against isolate AM2

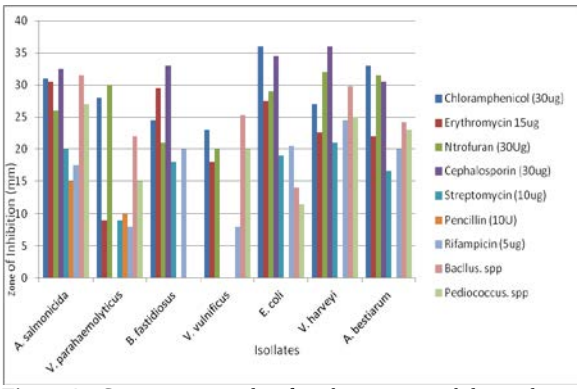

Figure 4. Comparative results of antibiotic susceptibility and probiotic efficacy against the identified pathogens**.**

4. Discussion

The maximum bacterial load was found to exist in the soil sample of the mixing water zone of Kolatali, Cox's Bazarand Sonapara, Cox's Bazar. The waste water of the hatchery, discharged with poor or no treatment, is supposed to be responsible for making the raw seawater contaminated. Wang and his co-workers published their studies on the total bacterial counts of new and three-year-old grow-out ponds for the cultivation of *Liptopenaeus vannamei*. Their findings revealed that the total bacterial count of a recently-constructed pond was 1.11×10^6 CFU/mL, while it was 6.25×10^6 CFU/mL for a three-year-old pound. Most of the Hatcheries' total bacterial count and total vibrio load count were found similar. The *Vibrio* count of treated water was found slightly lower for some hatcheries. The *Vibrio* species were *V. vulnificus*, *V. harveyi* and *V. parahaemolyticus* which are commonly termed as the pathogenic bacteria for shrimp larvae. The other identified bacteria have also detrimental effects for shrimp hatchery management. The root causes of these bacterial infections include the improper treatment of raw water and the insufficient storage conditions of storage tanks to maintain them as contamination-free. Moreover, in hatcheries, the algal culture tank constitutes another vital source of potential bacterial contamination where both the total bacterial count and total vibrio count were high. It is evident that *V. harveyi* is the most dominant pathogenic *Vibrio* species that has a greater effect on shrimp PL during the rearing period. According to Lavilla-Pitogo *et al*., (1998) and Karunasagar *et al*., (1994), Luminous bacteria, particularly *V. harveyi*, and occasionally other luminous species, have become recognized as a devastating pathogen of Penaeid shrimp larvae and adults throughout Southeast Asia. The salinity of this area facilitates the pathogenic *Vibrio* growth. This environment proved congenial for harmful bacterial species like *Vibrio harveyi, V. fisheri, V. spplendidus* and *V. vulnificus* for their survival and multiplication. To prevent diseases' outburst in shrimp hatcheries, the temperature of the rearing water tanks, in particular, needs to be maintained at optimum levels, and least fluctuations in temperature would lead to luminous vibriosis. Although motile aeromonads appropriately receive much notoriety as pathogens of fish, it is important to note that these bacteria also compose part of the normal intestinal microflora of healthy fish. Therefore, the presence of these bacteria, by themselves, is not indicative of a disease, and consequently, stress is often considered to be a contributing factor in the outbreaks of disease caused by these bacteria. In the present study, the bacterial genus *Aeromonas* was identified as the second most dominant bacteria in the shrimp culture system. The prevalence of this bacterium is an indication of its relation to pathogenic infections of cultured shrimp. Two of the other bacteria identified, namely *B. fastidiosus* and *E. coli* were also reported to be present in the shrimp culture system of which *Bacillus* spp. is used as the probiotic treatment in shrimp hatcheries to control other bacterial growth. Although *E. coli* is not so much reported in shrimp culture systems, the presence of *E. coli* is not unexpected due to the widespread availability of this organism which is also regarded as the pathogenic microbes affecting shrimp growth. All penaeid shrimp hatcheries encounter bacterial problems that impact the production. Antibiotic treatments to control pathogenic bacteria problems yield varying results. However, in the current research work, some of the antibiotics showed effective results in controlling bacterial growth in aquaculture. At present, the introduction of Probiotics, as 'bio-friendly agents' such as lactic acid bacteria and *Bacillus* spp. into the culture environment to control and compete with pathogenic bacteria as well as to promote the growth of the cultured organisms is gaining popularity. The present study has used the following antibiotics: Chloramphenicol, Erythromycin, Penicillin, Rifampicin, Nitrofuran, Cephalosporin, Streptomycin and some commercially available probiotics including *Bacillus.* spp, and *Pediococcus*. spp.. Both probiotics showed good results against all pathogens except *B. fastidiosus* because *B. fastidiosus* itself is a genus of the applied probiotic bacteria. Two common pathogens of shrimp hatcheries, namely *V. parahaemolyticus* and *V. vulnificus* exhibited resistance against the antibiotic cephalosporin and streptomycin, but showed significant zones of inhibition (22 mm against *Bacillus* spp., and 15 mm against *Pediococcus* spp. for *V. parahaemolyticus*, and 25.3 mm against *Bacillus* spp, and 20 mm against *Pediococcus* spp for *V. vulnificus*) against probiotics. This indicates that the presence of probiotic bacteria within the shrimp aquaculture can cause a significant decrease of pathogenic microorganisms through their antimicrobial action against a wide range of shrimp pathogens. With the use of antibiotics or disinfectants to kill bacteria, some bacteria survive (either strains of the pathogen or others) because they carry genes for resistance (Moriarty 1998). These will then grow rapidly because their competitors are removed.. Antibiotic-resistant bacterial strains develop and flourish over a short period of time. In contrast, Probiotic bacteria produce substances with bactericidal or bacteriostatic effects on other microbial populations (Servin 2004) such as bacteriocins, hydrogen peroxide, siderophores, lysozymes, proteases, among many others (Panigrahi 2007 and Tinh 2007). Besides, some bacteria produce organic acids and volatile fatty acids (e.g., lactic, acetic, butyric and propionic acids), that can result into the reduction of pH in the gastrointestinal lumen, thus, preventing the growth of opportunistic pathogenic microorganisms (Tinh 2007).

5. Conclusion

Waste water discharged from shrimp hatcheries and aquaculture without any or proper treatment is a potential source for microbial contamination within the shrimp culture. The untreated waste water gets mixed with seawater which is further used for hatchery operation. The representative microbial population within a shrimp culture includes the *Vibrio* spp., *Aeromonas* spp., *Bacillus fastidiosus* and *E. coli* among which *Vibrio* and *Aeromonas* are the pathogenic microorganisms which cause diseases to shrimp. The antibiotic effects against shrimp pathogens are strong enough to prevent any microbial growth; however, therapeutic regimen antibiotics used leave some negative impacts such as their residual toxicity, an emerging drug resistance, immunosuppression, and the reduction of consumers' preferences for drug-treated aquatic products in the market. Accordingly, the demand for non-antibiotic-based, and environmentally friendly agents is highly desired for health management in aquaculture. The use of probiotics is an effective alternative sustainable source of beneficial microbes with bactericidal or bacteriostatic effects against pathogenic bacteria, and with anti-bacterial, anti-viral, and anti-fungal activities. Further studies on the probiotic efficacy are still required to determine the appropriate dosage per unit of the aquaculture water system before commercial use.

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Reference

Bauer AW, Kirby MM, Sherris JC and Turuck M.1966. Antibiotic susceptibility testing by a standardized single disc method. *Am. J. Clin. Pathol*.,**45(4):**493-496

Buchanon RE and Gibson NE. 1974. **Bergey's Manual of Determinative Bacteriology**, 8th ed. Williams and Wilkins Co. Baltimore. pp. 530-534.

Holt JG, Krieg NR, Sneath PHA, Staley JT and Williams ST.2000. **Bergey's Manual of Determinative Bacteriology**, 9th (Ed.) Lippincott Williams and Wilkins, Baltimore. pp. 532,549-551,559,576,592.

Karunasagar I, Pai R, Malathi GR and Karunasagar I. 1994. Mass mortalities of Penaeus monodon larvae due to antibiotic resistant *Vibrio harveyi* infection. *Aquaculture*,**128**:203–209.

Lakshmi B, Viswanath B, Sai Gopal DVR. 2013. Probiotics as antiviral agents in shrimp aquaculture. *J Pathol*., Article ID 424123..

Lavilla-Pitago CR, Leano EM and Paner MG.1998. Mortalities of pond-cultured juvenile shrimp, Penaeus monodon, associated with dominance of luminescent *vibrios* in the rearing environment. *Aquaculture*,**164**:337–349

Magaldi S, Mata-Essayag S, Hartung De Capriles C, Perez C, Colella Mt, Olaizola C andOntiveros Y. 2004.Well diffusion for antifungal susceptibility testing. *Int J Infect Dis*.,**8(1):** 39-45.

Moriarty DJW. 1998. Control of luminous *Vibrio*species in penaeid aquaculture ponds. *Aquaculture*, **164**:351–358.

Newaj-Fyzul A and Austin B. 2015. Probiotics, immuno-stimulants, plant products and oral vaccines, and their role as feed supplements in the control of bacterial fish diseases. *J Fish Dis*., **38 (11):**937-955.

Ohhira ILT, Tamura N, Fujii K, Inagaki and Tanaka H. 1996. Antimicrobial activity against methicillin-resistant *Staphyhcoccus aureus*in the culture broth of *Enterococcus faecalis* TH10, an isolate from Malaysian fermentation food. *Temph. Japanese J. Dairy and Food Sci.*, **45:**93-96.

Panigrahi A and Azad IS. 2007. Microbial intervention for better fish health in aquaculture: the Indian scenario. *Fish Physiol Biochem.*, **33:**429–440

Sanders ER. 2012. Aseptic laboratory techniques: plating methods. *J. Vis. Exp*.**, 63**:1-18**.**

Servin A. 2004. Antagonistic activities of lactobacilli and bifidobacteria against microbial pathogens. *Microbiol Rev*., **28:**405–440.

Tinh NTN, Dierckens K, Sorgeloos P. 2007. A review of the functionality of probiotics in the larviculture food chain, *Mar Biotechnol*.,**10**: 1–12.

Verschuere L, Rombaut G, Sorgeloos P and Verstraete W. 2000. Probiotic bacteria as biological control agents in aquaculture. *Microbiol Mol Biol Rev*., **64(4):**655–671

Vijayan KK, Singh ISB and Jayaprakash NS. 2006. A brackish water isolate of *Pseudomonas* PS-102, a potential antagonistic bacterium against pathogenic vibrios in penaeid and non-penaeid rearing systems. *Aquaculture*, **251**:192–200.

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The Efficacy of *Alstonia boonei* Stembark Oil as a Long-term Storage Protectant against Cowpea Bruchid, *Callosobruchus maculatus* (Fab.) (Coleoptera: Chrysomelidae)

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Abstract

This study was conducted to assess the efficacy of *Alstonia boonei* stembark oil extracted with five solvents (methanol, ethanol, acetone, petroleum ether, and n-hexane) as a long-term storage protectant (after thirty, sixty, and ninety days of treatment) against *Callosobruchus maculatus* in the Laboratory. The mortality of adult insects, oviposition, percentage of adult emergence, progeny development, seed damage, weight loss, and beetle perforation index were measured and studied in this research. The results showed that the n-hexane oil extract of the *A. boonei* stembark was the most toxic, which caused 45 %, 57.5 %, 67.5 % and 75 % of adult mortality of *C. maculatus* at the rates of 1 %, 2 %, 3 % and 4 %/ 20g of cowpea seeds after thirty days of treatment respectively. It was followed by the petroleum ether oil extract, while the least toxic oil was the acetone extract. Generally, the percentage of adult mortality of *C. maculatus* decreased with the increase of storage periods (sixty and ninety days). The *Alstonia boonei* stembark oil extracted with non-polar and polar solvents completely inhibited the perforation potential of bruchids. The extracted oil can definitely serve as a biopesticide for the protection of cowpea seeds in storage up to ninety days against infestation by *C. maculatus*.

Keywords: *Alstonia boonei*, Perforation index; Progeny development, *Callosobruchus maculates*, cowpeas, Protectant.

1. Introduction

Postharvest losses of cowpea seeds by their major coleopteran insect pest, *Callosobruchus maculatus*, has led to seed perforation, reductions in weight, loss of nutritional value, market value, and viability (Ofuya, 2001; Akinkurolere, 2012; Idoko, 2016). Cowpea seeds are considered by farmers of poor resources in the tropical regions as the poor man's meat to combat malnutrition in young children instead of expensive protein sources such as meat, fish, and eggs, Cowpea seeds can face up to 100 % losses in terms of qualities and quantities as a result of *C. maculatus* infestation (Singh, 1985; Ogunwolu and Odunlami, 1996; Akinkurolere *et al*., 2006; Akinkurolere, 2012; Ileke, 2014).

Cowpea bruchid is a field-to-store coleopteran insect pest. Their eggs are laid on the cowpea pods by adult females before harvest and these can develop into larvae that feed exclusively on the pods after they penetrate through the pod covers and remain concealed within the seeds (Southgate, 1978, Akinkurolere, 2012). During harvest, the seeds infested with bruchid developmental stages are conveyed to store where infestation continues, and the emergence of adult *C. maculatus* leads to secondary infestation such as fungi causing a total destruction of the seeds' viability within three to four months (Singh and Jackai, 1985; Akinkurolere, 2012).

In order to reduce the qualitative and quantitative losses, the management of *C. maculatus* by Nigerian farmers has been dominated by the use of synthetic chemical insecticides and fumigants (Park *et al*., 2003; Akinkurolere, 2012; Idoko and Adesina 2012; Ileke *et al*., 2016). The use of synthetic chemical insecticides in the developing countries is restricted by environmental, financial, and safety contemplations. The high cost of chemical insecticides has led to the indiscriminate use of cheap pesticides of high mammalian toxicity to grains by farmers and traders in most Nigerian markets, which exposes the consumers of such products to chronic toxicity (Akinkurolere, 2012). The indiscriminate use of synthetic pesticides by untrained local farmers and traders has been a major concern for agricultural and storage entomologists all over the world who wish to find alternative methods that are readily available, eco-friendly, and cheap in order to replace the chemical insecticides (Adedire and Lajide 1999; Ogunwolu and Odunlami, 1996; Odeyemi *et al*., 2006; Ileke *et al*., 2012; 2013; 2016). The use of botanicals as pesticides in order to solve the problems of high cost, environmental hazards and the killing of the natural enemies of the pests is gaining more attention. Recently, researches have revealed that plant powders, ashes, oils, extracts, and the latex of different plant parts are effective protectants of stored cowpeas (Adedire and Lajide 1999; Lale and Abdulrahman, 1999; Akinkurolere *et al*., 2006;

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Akinkurolere, 2007; Ileke 2014; Ileke *et al*., 2013; 2014; Okosun and Adedire 2010; 2017).

Alstonia boonei belongs to the family Apocyanaceae. It is an African large evergreen deciduous crude medicinal tree that sheds its leaves annually. The plant is about 45 m tall, and its trunk is 1.2 m in diameter. The plant stembark and latex are applied in traditional medicine for treating many diseases (Moronkola and Kunle, 2012). In traditional African medicine, *A. boonei* is a medicinal plant used extensively for the treatment of malaria, fever, intestinal helminths, rheumatism, hypertension (Terashima, 2003; Betti, 2004; Abel and Busia, 2005). The insecticidal activity of *A. boonei* has been reported by several workers (Ileke and Oni 2011; Ileke *et al*., 2012; Ileke *et al*., 2013; 2014). Ileke and Oni (2011) reported the insecticidal potential of *A. boonei* stembark powder against *Sitophilus zeamais*. Ileke *et al*. (2012 and 2013) reported the insecticidal activity of *A. boonei* powder against *C. maculatus* and the response of cowpea bruchid to a 2 % of *A. boonei* stembark oils extracted with methanol, ethanol, acetone, petroleum ether, and n-hexane using cold extraction methods. Ileke *et al*. (2014) reported the insecticidal activity of *A. boonei* latex against *C. maculatus*.

Literature on the use of *A. boonei* stembark oils extracted with methanol, ethanol, acetone, petroleum ether, and n-hexane as long-term storage protectants against cowpea bruchid is relatively scarce. The aim of this research is to evaluate the *A. boonei* stembark oil extracted with five solvents as a long-term storage protectant (after thirty, sixty and ninety days of treatment) against *C. maculatus*.

2. Materials and Methods

2.1. Insect Rearing

The adults of the cowpea bruchid, *C. maculatus*, were supplied by Storage Entomology Research Laboratory, Department of Biology, Federal University of Technology, Akure, Nigeria. Eighty pairs of *C. maculatus* were introduced into a 1L glass kilner jar containing 300g of *Vigna unguiculata* (cultivar Ife brown) obtained from the International Institute for Tropical Agriculture, Ibadan, Nigeria. The beetle colony was maintained under constant insectary conditions of 28±2°C and 75±5 % relative humidity.

2.2. Plant Materials

The fresh stembark of *A. boonei* stem was obtained from Akola farm, Igbara Odo Ekiti, Nigeria. The plant stembark was first authenticated by a plant taxonomist at the Department of Crop, Soil, and Pest Management, Federal University of Technology, Akure, Nigeria. The stembark was air- dried in the Laboratory for four weeks before it was pulverized into fine powder using an electric blender, and was sieved using a $1mm^2$ perforation sieve. The powder was kept in plastic containers with tight lids and was stored in a refrigerator at 4°C prior to use.

2.3. Soxhlet Extraction of A. boonei Stembark.

Three hundred grams (300g) of the powdered stembark was separately extracted with methanol, ethanol, acetone, petroleum ether, and n-hexane using the Soxhlet extraction method. The excess solvent was recovered using a rotary evaporator vacuum. The resulting oil was concentrated by air-drying to remove the traces of the solvent. From this stock solution, different oil concentrations (1 %, 2 %, 3 % and 4 %) were prepared separately.

2.4. Contact Toxicity of A. boonei Stembark Oil

Twenty grams (20g) of cowpea seeds that have been previously treated for thirty (30), sixty (60) and ninety (90) days with different concentrations (1 %, 2 %, 3 % and 4 %) of *A. boonei* stembark oils were used for this study. Ten pairs of two – three-day-old adults of *C. maculatus* were introduced to each of the containers and covered. Four replicates of the treated and untreated controls were laid out in Complete Randomized Design. The adult mortality was assessed after twenty-four hours. The adults were considered dead when probed gently with a fine needle and showed no response. At the end of day one, all insects, both dead and alive, were removed from each container, and the eggs were counted and recorded before returning the seeds to their respective containers.

The experimental setup was kept inside the insect-rearing cage for thirty more days for the emergence of the first filial (F_1) generation. The containers were sieved out, and the newly- emerged adult cowpea bruchids were counted and recorded. The percentage of adult emergence was calculated as described by Odeyemi and Daramola (2000):

$$\% \text{ Adult emergence} = \frac{\text{Total number of adult emergence}}{\text{Total number of larvae introduced}} \text{ x } \frac{100}{1}$$

The percentage of reduction in adult emergence of F_1 progeny or inhibition rate (IR) was calculated according to the method described by Tapondju *et al*. (2002):

$$\% \text{ IR} = \frac{C_n - T_n}{C_n} \text{ x } \frac{100}{1}$$

where C_n is the number of emerged insects in the control. and T_n is the number of emerged insects in the treated container.

The percentage of weight loss of the cowpea seeds was also determined:

$$\% \text{ Weight loss} = \frac{\text{Change in weight}}{\text{Initial weight}} \text{ x } \frac{100}{1}$$

The numbers of damaged cowpea seeds were also evaluated by counting wholesome seeds and the seeds with bruchid emergence holes:

$$\% \text{ Seed damage} = \frac{\text{Number of seeds damaged}}{\text{Total number of seeds}} \text{ x } \frac{100}{1}$$

The percentage of seeds' damage was calculated using a standard method. Beetle Perforation Index (BPI) used by Fatope *et al.* (1995) was adopted for the analysis of damage. Beetle perforation index (BPI) was defined as follows:

$$\text{BPI} = \frac{\% \text{ treated cowpea seeds perforated}}{\% \text{ control cowpea seeds perforated}} \text{ x } \frac{100}{1}$$

BPI value exceeding fifty has been regarded as enhancement of infestation by the weevil or negative protectability of the extract tested.

2.5. Statistical Analysis

The mortality percentages were calculated and corrected relative to the associated controls using Abbott's (1925) formula**.** Data were subjected to analysis of

variance (ANOVA), and means were separated using the new Duncan's Multiple Range Test.

3. Results

3.1. Mortality of Adult C. maculatus in Treated Cowpeas

Table 1 presents the toxicity of *A. boonei* stembark oils after thirty, sixty, and ninety days of treatment of adult mortality of *C. maculatus*.

Table 1. Dose response mortality % of *C. maculatus* adults treated with *A. boonei* stembark oils after 30, 60 and 90 days of treatment.

Oils of *A. boonei* extracted by	Concentration in %	Mean % Mortality ± S.E. after 30-90 Days		
		30	60	90
Methanol	1	27.50±2.50^{b}	25.00±2.89^{b}	20.00±4.08^{b}
Ethanol		25.00±2.89^{b}	22.50±7.50^{b}	17.50±2.50^{b}
Acetone		17.50±2.50^{b}	15.0±2.89^{b}	12.50±3.74^{b}
Petroleum ether		32.50±7.50^{b}	30.00±2.89^{b}	27.50±2.50^{b}
N-hexane		45.00±2.89^{b}	37.50±2.50^{b}	32.50±7.50^{b}
Control	0.0	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}
Methanol	2	47.50±2.50^{c}	40.00±4.08^{b}	30.00±4.08^{b}
Ethanol		37.50±2.89^{bc}	32.50±7.50^{b}	25.00±2.89^{b}
Acetone		30.00±4.08^{b}	27.50±2.50^{b}	22.50±7.50^{b}
Petroleum ether		55.00±2.89^{c}	42.50±7.50^{bc}	35.00±2.89^{b}
N-hexane		57.50±2.50^{c}	50.00±5.79^{c}	37.50±2.50^{b}
Control	0.0	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}
Methanol	3	50.00±5.79^{c}	40.00±4.08^{bc}	30.00±4.08^{b}
Ethanol		47.50±2.50^{bc}	35.00±2.89^{b}	27.50±2.50^{b}
Acetone		37.50±2.50^{b}	30.00±4.08^{b}	22.50±7.50^{b}
Petroleum ether		57.50±2.50^{cd}	47.50±2.50^{c}	37.50±2.50^{b}
N-hexane		67.50±2.50^{d}	52.50±7.50^{c}	40.00±4.08^{b}
Control	0.0	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}
Methanol	4	62.50±7.50^{bc}	42.50±7.50^{bc}	30.00±4.08^{b}
Ethanol		60.00±4.08^{bc}	37.50±2.50^{bc}	27.50±2.50^{b}
Acetone		47.50±2.50^{b}	32.50±7.50^{b}	25.00±2.89^{b}
Petroleum ether		57.50±2.50^{c}	50.00±5.79^{c}	37.50±2.50^{b}
N-hexane		75.00±2.89^{c}	55.00±2.89^{c}	42.50±7.50^{b}
Control	0.0	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}

Each value is a mean ± standard error of four replicates. Means followed by the same letter along the column are not significantly different (P>0.05) using New Duncan's Multiple Range Test.

The n-hexane oil extract of *A. boonei* stembark caused 45%, 57.5%, 67.5% and 75 % of adult mortality of *C. maculatus* at the rates of 1 %, 2 %, 3 % and 4 %/ 20g of cowpea seeds after thirty days of treatment respectively. This is followed by the petroleum ether oil extract of *A. boonei* stembark which evoked 32.5 %, 55 %, 57.5 % and 67.5 % of the mortality of cowpea bruchid at the rates of 1 %, 2 %, 3 % and 4 % / 20g of cowpea seeds after thirty days of treatment respectively. The least toxic oil was the stembark oil extracted with acetone which evoked 17.5 %, 30 %, 37.5 % and 47.5 % of the mortality of adult *C. maculatus* at the rates of 1 %, 2 %, 3 % and 4 % / 20g of cowpea seeds after thirty days of treatment respectively.

Sixty days post-treatment, 37.5 %, 50 %, 52.5 % and 55 % rates of adult mortality of the cowpea bruchids were recorded on cowpea seeds treated with the n-hexane oil extract of *A. boonei* stembark at the rates of 1 %, 2 %, 3 % and 4 % respectively. Ninety days post-treatment, the rates 32.5 %, 37.5 %, 40 % and 42.5 % of adult mortality of the cowpea bruchids were recorded on the seeds treated with the n-hexane oil extract of *A. boonei* stembark at the rates of 1 %, 2 %, 3 % and 4 % respectively. On the whole, the percentage of adult mortality of *C. maculatus* decreased with the increase of the storage period.

3.2. Effect of Treatments on C. maculatus Emergence

The effects of *A. boonei* stembark oils after thirty, sixty, and ninety days of treatment on oviposition, adult emergence, and reduction in progeny development of the adults of *C. maculatus* are presented in Tables 2, 3, 4, and 5. In all cases, the ANOVA results showed that the treatments had significant effects (P < 0.05) against the emergence of the first filial generation of *C. maculatus*, with the exception of the control groups. Thirty days post-treatment, the methanol, ethanol, petroleum ether, and n-hexane stembark oils reduced the number of eggs laid by cowpea bruchids showing a 100 % reduction in progeny development of adult bruchids at all of the tested concentration rates (Tables 2, 3, 4, and 5).

Sixty days post-treatment, the methanol, ethanol, petroleum ether, and n-hexane stembark oils at the rate of 4 % reduced the number of eggs laid by cowpea bruchid, showing a 100 % reduction rate in progeny development of adult bruchids, while the acetone extract of *A. boonei* stembark showed 12.12 % of adult emergence and a 95 % inhibition rate of progeny development of cowpea bruchid (Table 5). The number of eggs laid, the percentage of adult emergence, and progeny development of adult *C. maculatus* all decreased as the extract concentrations increased (Tables 2, 3, 4, and 5).

Table 2. Number of eggs laid, adult emergence and inhibition rate (IR) of adult *C. maculatus* in cowpea seeds treated with 1% oil of *A. boonei* stembark after 30, 60 and 90 days of treatment.

Days after treatment	1% oil of *A. boonei*	Mean number of eggs laid ± SE	% adult emergence ± SE	% IR ± SE
30	Methanol	8.50±1.23^{a}	0.00±0.00^{a}	100.00±0.00^{g}
	Ethanol	8.75±0.85^{a}	0.00±0.00^{a}	100.00±0.00^{g}
	Acetone	10.25±1.70^{ab}	29.27±1.40^{c}	82.35±3.50^{cde}
	Pet-ether	8.50±1.23^{a}	0.00±0.00^{a}	100.00±0.00^{g}
	N-hexane	8.00±0.91^{a}	0.00±0.00^{a}	100.00±0.00^{g}
	Untreated	22.25±2.70^{b}	76.41±3.25^{f}	0.00±0.00^{a}
60	Methanol	9.00±0.91^{a}	11.11±0.43^{b}	95.00±2.89^{fg}
	Ethanol	9.25±1.70^{a}	21.62±1.67^{bc}	90.00±4.08^{efg}
	Acetone	11.00±0.91^{ab}	27.27±1.40^{c}	85.00±2.89^{def}
	Pet-ether	9.00±0.91^{a}	11.11±0.43^{b}	95.00±2.89^{fg}
	N-hexane	8.75±0.85^{a}	11.43±0.74^{b}	95.00±2.89^{fg}
	Untreated	25.00±2.89^{b}	80.00±4.08^{f}	0.00±0.00^{a}
90	Methanol	10.00±0.91^{a}	40.00±4.08^{de}	77.78±3.12^{cd}
	Ethanol	10.50±1.23^{ab}	47.62±2.53^{e}	72.22±3.41^{c}
	Acetone	11.25±1.70^{ab}	71.11±3.43^{f}	55.56±2.65^{b}
	Pet-ether	10.00±0.91^{a}	40.00±4.08^{de}	77.78±2.12^{cd}
	N-hexane	9.75±0.85^{a}	30.77±4.12^{cd}	83.33±3.45^{cde}
	Untreated	23.00±2.96^{b}	78.26±3.38^{f}	0.00±0.00^{a}

Each value is a mean ± standard error of four replicates. Means followed by the same letter along the column are not significantly different (P>0.05) using New Duncan's Multiple Range Test.

Table 3. Number of eggs laid, adult emergence and inhibition rate (IR) of adult *C. maculatus* in cowpea seeds treated with 2% oil of *A. boonei* stembark after 30, 60 and 90 days of treatment.

Days after treatment	2% oil of *A. boonei*	Mean number of eggs laid ± SE	% adult emergence ± SE	% IR ± SE
30	Methanol	7.50±1.23^{a}	0.00±0.00^{a}	100.00±0.00^{e}
	Ethanol	7.75±0.85^{a}	0.00±0.00^{a}	100.00±0.00^{e}
	Acetone	9.50±1.23^{ab}	21.05±1.40^{bc}	88.35±3.20^{d}
	Pet-ether	7.50±1.23^{a}	0.00±0.00^{a}	100.00±0.00^{e}
	N-hexane	7.25±1.70^{a}	0.00±0.00^{a}	100.00±0.00^{e}
	Untreated	22.25±2.70^{b}	76.41±3.25^{f}	0.00±0.00^{a}
60	Methanol	8.75±0.85^{a}	11.43±0.74^{b}	95.00±2.89^{de}
	Ethanol	9.00±0.91^{a}	11.62±0.43^{b}	90.00±4.08^{de}
	Acetone	10.75±0.85^{ab}	37.21±2.39^{d}	85.00±2.89^{d}
	Pet-ether	9.75±0.85^{ab}	10.26±1.02^{b}	95.00±2.89^{e}
	N-hexane	8.25±1.70^{a}	0.00±0.00^{a}	100.00±0.00^{e}
	Untreated	25.00±2.89^{b}	80.00±4.08^{f}	0.00±0.00^{a}
90	Methanol	9.75±0.85^{a}	30.77±4.16^{cd}	83.33±3.35^{cd}
	Ethanol	10.25±1.70^{ab}	39.02±3.91^{de}	72.78±2.12^{bc}
	Acetone	11.00±0.91^{ab}	54.55±2.83^{e}	66.67±2.31^{b}
	Pet-ether	10.00±0.91^{a}	30.00±4.08^{cd}	83.33±3.35^{cd}
	N-hexane	9.75±0.85^{a}	20.51±4.20^{bc}	88.89±3.27^{de}
	Untreated	23.00±2.96^{b}	78.26±3.38^{f}	0.00±0.00^{a}

Each value is a mean ± standard error of four replicates. Means followed by the same letter along the column are not significantly different (P>0.05) using New Duncan's Multiple Range Test.

Table 4. Number of eggs laid, adult emergence and inhibition rate (IR) of adult *C. maculatus* in cowpea seeds treated with 3% oil of *A. boonei* stembark after 30, 60 and 90 days of treatment.

Days after treatment	3% oil of *A. boonei*	Mean number of eggs laid ± SE	% adult emergence ± SE	% IR ± SE
30	Methanol	7.25±1.70^{a}	0.00±0.00^{a}	100.00±0.00^{f}
	Ethanol	7.50±1.23^{a}	0.00±0.00^{a}	100.00±0.00^{f}
	Acetone	9.25±1.70^{ab}	10.81±1.40^{b}	94.12±2.63^{cdef}
	Pet-ether	7.00±0.91^{a}	0.00±0.00^{a}	100.00±0.00^{f}
	N-hexane	6.75±0.85^{a}	0.00±0.00^{a}	100.00±0.00^{f}
	Untreated	22.25±2.70^{bc}	76.41±3.25^{e}	0.00±0.00^{a}
60	Methanol	8.00±0.91^{a}	0.00±0.00^{a}	100.00±0.00^{f}
	Ethanol	8.00±0.91^{a}	12.50±1.23^{b}	90.00±4.08^{d}
	Acetone	9.00±0.91^{a}	22.22±2.41^{bc}	85.00±2.89^{cde}
	Pet-ether	7.75±0.85^{a}	0.00±0.00^{a}	100.00±0.00^{f}
	N-hexane	7.25±1.70^{a}	0.00±0.00^{a}	100.00±0.00^{f}
	Untreated	25.00±2.89^{c}	80.00±4.08^{e}	0.00±0.00^{a}
90	Methanol	9.00±0.91^{a}	22.77±2.41^{bc}	88.89±3.22^{cd}
	Ethanol	9.25±1.23^{a}	32.43±2.74^{c}	83.33±3.35^{bcd}
	Acetone	10.00±0.91^{ab}	50.00±5.79^{d}	72.22±3.41^{b}
	Pet-ether	9.00±0.91^{a}	11.11±0.58^{b}	94.44±3.62^{cdef}
	N-hexane	8.75±0.85^{a}	11.43±0.74^{b}	94.44±3.62^{cdef}
	Untreated	23.00±2.96^{bc}	78.26±3.38^{e}	0.00±0.00^{a}

Each value is a mean ± standard error of four replicates. Means followed by the same letter along the column are not significantly different (P>0.05) using New Duncan's Multiple Range Test.

Ninety days post-treatment, the petroleum ether and n-hexane stembark oils at the concentration rate of 4 % reduced the number of eggs laid by cowpea bruchids showing a 100 % reduction rate in the progeny development of adult bruchids, while the methanol, ethanol, and acetone extracts of *A. boonei* stembark oils allowed 11.43 %, 11.11 %, and 32.43 % of adult emergence and 94.44 %, 94.44 % and 83.33 % inhibition or reduction rates of progeny development of cowpea bruchid respectively (Table 5).

Table 5. Number of eggs laid, adult emergence and inhibition rate (IR) of adult *C. maculatus* in cowpea seeds treated with 4% oil of *A. boonei* stembark after 30, 60 and 90 days of treatment.

Days after treatment	4% oil of *A. boonei*	Mean number of eggs laid ± SE	% adult emergence ± SE	% IR ± SE
30	Methanol	6.25±1.70^{a}	0.00±0.00^{a}	100.00±0.00^{c}
	Ethanol	6.25±1.70^{a}	0.00±0.00^{a}	100.00±0.00^{c}
	Acetone	7.25±1.70^{a}	0.00±0.00^{a}	100.00±0.00^{c}
	Pet-ether	6.00±0.91^{a}	0.00±0.00^{a}	100.00±0.00^{c}
	N-hexane	5.75±0.85^{a}	0.00±0.00^{a}	100.00±0.00^{c}
	Untreated	22.25±2.70^{bc}	76.41±3.25^{d}	0.00±0.00^{a}
60	Methanol	7.00±0.91^{a}	0.00±0.00^{a}	100.00±0.00^{c}
	Ethanol	7.50±1.23^{a}	0.00±0.00^{a}	100.00±0.00^{c}
	Acetone	8.25±1.70^{a}	12.12±1.63^{b}	95.00±2.89^{bc}
	Pet-ether	7.00±0.91^{a}	0.00±0.00^{a}	100.00±0.00^{c}
	N-hexane	6.75±0.85^{a}	0.00±0.00^{a}	100.00±0.00^{c}
	Untreated	25.00±2.89^{c}	80.00±4.08^{d}	0.00±0.00^{a}
90	Methanol	8.75±0.85^{a}	11.43±0.74^{b}	94.44±3.62^{bc}
	Ethanol	9.00±0.91^{a}	11.11±0.58^{b}	94.44±3.62^{bc}
	Acetone	9.25±1.70^{ab}	32.43±2.74^{c}	83.33±3.35^{b}
	Pet-ether	8.25±1.70^{a}	0.00±0.00^{a}	100.00±0.00^{c}
	N-hexane	8.00±0.91^{a}	0.00±0.00^{a}	100.00±0.00^{c}
	Untreated	23.00±2.96^{bc}	78.26±3.38^{d}	0.00±0.00^{a}

Each value is a mean ± standard error of four replicates. Means followed by the same letter along the column are not significantly different (P>0.05) using New Duncan's Multiple Range Test.

3.3. Beetle Perforation Index caused by C. maculatus

The percentage of seeds' damage, weight loss, and Beetle Perforation Index caused by *C. maculatus* in cowpea seeds treated with *A. boonei* stembark oils after thirty, sixty and ninety days of treatment are shown in Table 6, 7, 8, and 9. Thirty days post-treatment, the methanol, ethanol, petroleum ether, and n-hexane of stembark oils completely protected the seeds from being damaged by cowpea bruchids at all the concentrations tested. There was neither seed damage nor weight loss observed in the cowpea seeds treated with the acetone oil of *A. boonei* stembark and BPI was zero for the concentrations tested after thirty days of application (Tables 6, 7, 8, and 9).

Sixty days post-treatment, the methanol, petroleum ether, and n-hexane of the stembark oil extracts completely protected the seeds from being damaged by cowpea bruchid at the rates of 2%, 3%, and 4%.

Ninety days post-treatment, only the n-hexane oil completely protected the cowpea seeds from being damaged by *C. maculatus*. The n-hexane oil effect was not significantly different from the petroleum oil extract of *A. boonei*. Generally, the percentage of seed damage, weight loss, and Beetle Perforation Index by *C. maculatus* increased with increase of the storage period. Conversely, the percentages of seed damage, weight loss, and Beetle

Perforation Index by *C. maculatus* decreased with the increase in the oil concentrations.

Table 6. Perforation Index caused by *C. maculatus* in cowpea seeds treated with 1% oil of *A. boonei* stembark oil after 30, 60 and 90 days of treatment.

Days after treatment	1% oil of *A. boonei*	Mean total number of cowpea seeds	Mean number of damaged cowpea seeds	Mean % cowpea seeds damaged	Mean % weight loss	Beetle perforation Index (BPI)*
30	Methanol	93.00	0.00	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}
	Ethanol	94.75	0.00	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}
	Acetone	94.00	3.25	3.46±0.11^{ab}	6.24±0.68^{b}	18.39±1.16^{c}
	Pet-ether	95.25	0.00	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}
	N-hexane	92.75	0.00	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}
	Untreated	93.00	17.50	18.81±1.19^{c}	62.52±2.21^{c}	50.00±0.00^{e}
60	Methanol	93.75	1.00	1.07±0.54^{ab}	3.07±0.54^{ab}	5.06±0.73^{b}
	Ethanol	94.25	1.50	1.59±0.11^{ab}	3.70±0.97^{ab}	7.52±1.21^{b}
	Acetone	94.00	4.00	4.26±0.57^{b}	7.38±0.18^{b}	20.15±4.74^{c}
	Pet-ether	95.00	1.00	1.05±0.42^{ab}	2.92±0.87^{ab}	4.97±0.95^{b}
	N-hexane	92.75	1.00	1.08±0.76^{ab}	3.11±0.61^{ab}	5.11±0.43^{b}
	Untreated	94.75	20.25	21.14±2.66^{c}	65.68±3.83^{c}	50.00±0.00^{e}
90	Methanol	94.50	4.25	4.50±0.23^{b}	7.74±0.83^{ab}	23.24±2.63^{c}
	Ethanol	93.00	5.50	5.91±1.19^{b}	7.80±0.23^{ab}	30.53±4.74^{cd}
	Acetone	94.00	8.00	8.51±1.91^{b}	9.63±0.61^{b}	43.96±2.96^{de}
	Pet-ether	95.00	4.75	5.00±0.91^{b}	7.29±0.46^{ab}	25.83±2.82^{c}
	N-hexane	93.75	3.50	3.73±0.86^{ab}	6.34±1.44^{ab}	19.27±3.40^{c}
	Untreated	94.25	18.25	19.36±0.62^{c}	63.67±3.08^{c}	50.00±0.00^{e}

Each value is a mean ± standard error of four replicates. Means followed by the same letter along the column are not significantly different (P>0.05) using New Duncan's Multiple Range Test.

*****Beetle Perforation Index (BPI). Value lower than 50 is an indication of positive protectant effect while BPI greater than 50 is an indication of negative protectability.

Table 7. Perforation Index caused by *C. maculatus* in cowpea seeds treated with 2% oil of *A. boonei* stembark after 30, 60 and 90 days of treatment

Days after treatment	2% oil of *A. boonei*	Mean total number of cowpea seeds	Mean number of damaged cowpea seeds	Mean % cowpea seeds damaged	Mean % weight loss	Beetle perforation Index (BPI)*
30	Methanol	94.25	0.00	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}
	Ethanol	93.75	0.00	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}
	Acetone	95.00	2.50	2.63±0.61^{ab}	4.99±0.72^{b}	13.39±1.67^{bc}
	Pet-ether	93.50	0.00	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}
	N-hexane	94.75	0.00	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}
	Untreated	95.00	17.50	18.81±1.19^{c}	62.52±2.21^{c}	50.00±0.00^{f}
60	Methanol	93.50	1.25	1.34±0.41^{ab}	3.62±0.59^{ab}	6.34±1.41^{b}
	Ethanol	94.00	1.75	1.86±0.19^{ab}	3.96±0.95^{ab}	8.80±1.20^{bc}
	Acetone	95.25	4.25	4.46±0.15^{b}	7.89±1.22^{b}	21.10±4.98^{cd}
	Pet-ether	94.00	1.25	1.33±0.03^{ab}	3.55±0.11^{ab}	6.29±1.39^{b}
	N-hexane	93.00	0.00	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}
	Untreated	94.75	20.25	21.14±2.66^{c}	65.68±3.83^{c}	50.00±0.00^{f}
90	Methanol	93.00	3.50	3.76±0.11^{ab}	4.74±0.54^{ab}	19.42±2.75^{d}
	Ethanol	92.75	4.25	4.58±0.13^{b}	4.71±0.97^{ab}	23.66±3.79^{cd}
	Acetone	94.25	6.50	6.90±1.29^{b}	7.38±0.18^{b}	35.64±2.59^{e}
	Pet-ether	95.00	3.00	3.16±0.37^{ab}	3.92±0.87^{ab}	16.32±2.74^{cd}
	N-hexane	94.25	2.25	2.39±0.61^{ab}	3.19±0.61^{ab}	12.35±3.20^{bcd}
	Untreated	94.25	18.25	19.36±0.62^{c}	63.67±3.08^{c}	50.00±0.00^{f}

Each value is a mean ± standard error of four replicates. Means followed by the same letter along the column are not significantly different (P>0.05) using New Duncan's Multiple Range Test.

*****Beetle Perforation Index (BPI). Value lower than 50 is an indication of positive protectant effect while BPI greater than 50 is an indication of negative protectability.

Table 8. Perforation Index caused by *C. maculatus* in cowpea seeds treated with 3% oil of *A. boonei* stembark after 30, 60 and 90 days of treatment

Days after treatment	3% oil of *A. boonei*	Mean total number of cowpea seeds	Mean number of damaged cowpea seeds	Mean % cowpea seeds damaged	Mean % weight loss	Beetle perforation Index (BPI)*
30	Methanol	94.00	0.00	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}
	Ethanol	92.25	0.00	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}
	Acetone	94.00	1.25	1.33±0.35^{ab}	3.55±0.11^{ab}	7.07±0.54^{b}
	Pet-ether	93.75	0.00	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}
	N-hexane	92.75	0.00	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}
	Untreated	95.00	17.50	18.81±1.19^{c}	62.52±2.21^{c}	50.00±0.00^{e}
60	Methanol	95.00	0.00	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}
	Ethanol	94.25	1.00	1.06±0.51^{ab}	3.47±0.14^{ab}	5.01±0.84^{b}
	Acetone	92.75	2.50	2.70±0.97^{ab}	4.96±0.94^{b}	12.77±1.81^{bc}
	Pet-ether	94.75	0.00	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}
	N-hexane	93.25	0.00	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}
	Untreated	94.75	20.25	21.14±2.66^{c}	65.68±3.83^{c}	50.00±0.00^{e}
90	Methanol	94.75	2.25	2.38±0.18^{ab}	3.11±0.43^{ab}	12.29±1.39^{bc}
	Ethanol	94.00	3.75	3.99±0.67^{ab}	3.96±0.23^{ab}	20.61±4.58^{cd}
	Acetone	93.50	5.50	5.88±1.23^{b}	6.59±0.09^{b}	30.37±4.15d
	Pet-ether	92.75	1.50	1.62±0.59^{ab}	3.80±0.21^{ab}	8.37±1.15^{b}
	N-hexane	95.00	1.25	1.36±0.07^{ab}	2.60±0.13^{ab}	7.03±1.64^{b}
	Untreated	94.25	18.25	19.36±0.62^{c}	63.67±3.08^{c}	50.00±0.00^{e}

Each value is a mean ± standard error of four replicates. Means followed by the same letter along the column are not significantly different (P>0.05) using New Duncan's Multiple Range Test.

*****Beetle Perforation Index (BPI). Value lower than 50 is an indication of positive protectant effect while BPI greater than 50 is an indication of negative protectability.

Table 9. Perforation Index caused by *C. maculatus* in cowpea seeds treated with 4% oil of *A. boonei* stembark after 30, 60 and 90 days of treatment

Days after treatment	4% oil of *A. boonei*	Mean total number of cowpea seeds	Mean number of damaged cowpea seeds	Mean % cowpea seeds damaged	Mean % weight loss	Beetle perforation Index (BPI)*
30	Methanol	92.75	0.00	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}
	Ethanol	93.50	0.00	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}
	Acetone	93.00	0.00	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}
	Pet-ether	94.00	0.00	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}
	N-hexane	93.25	0.00	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}
	Untreated	95.00	17.50	18.81±1.19^{b}	62.52±2.21^{c}	50.00±0.00^{d}
60	Methanol	94.00	0.00	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}
	Ethanol	93.50	0.00	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}
	Acetone	93.00	1.50	1.61±0.58^{a}	3.83±0.29^{ab}	7.61±1.58^{b}
	Pet-ether	92.75	0.00	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}
	N-hexane	95.00	0.00	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}
	Untreated	94.75	20.25	21.14±2.66^{b}	65.68±3.83^{c}	50.00±0.00^{d}
90	Methanol	93.00	1.50	1.61±0.58^{a}	3.83±0.29^{ab}	8.32±1.41^{bc}
	Ethanol	94.00	1.75	1.85±0.19^{a}	3.96±0.23^{ab}	9.61±1.58^{bc}
	Acetone	95.00	3.50	3.68±0.82^{a}	4.59±0.09^{b}	19.01±2.89^{c}
	Pet-ether	93.00	1.00	1.08±0.97^{a}	2.11±0.43^{ab}	5.58±0.13^{b}
	N-hexane	93.75	0.00	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}
	Untreated	94.25	18.25	19.36±0.62^{b}	63.67±3.08^{c}	50.00±0.00^{d}

Each value is a mean ± standard error of four replicates. Means followed by the same letter along the column are not significantly different (P>0.05) using New Duncan's Multiple Range Test.

*****Beetle Perforation Index (BPI). Value lower than 50 is an indication of positive protectant effect while BPI greater than 50 is an indication of negative protectability.

4. Discussion

Entomologists employed many procedures to screen plant materials for their efficacy against cowpea bruchid, *C. maculatus* (Adedire and Lajide, 1999; Ogunwolu and Odunlami, 1996; Okonkwo and Okoye, 1996; Akinkurolere, 2016; Ileke, 2014). In all of the tested procedures, efficacious materials adversely affected the beetles by killing them, at the adult, pupal and larval stages, exterminated oviposited eggs, or prevented the full expression of oviposition through antifeedants, fumigants, repellents, attractants and contact poisoning (Ogunwolu and Odunlami, 1996; Boeke *et al*., 2001 Akinkurolere et al., 2006; Akinkurolere, 2012).

The results of this study show that the n-hexane oil extract from *A. boonei* stembark with the lowest beetle perforation index, was the most effective against *C. maculatus*, showing the highest bruchid mortality, suppressing F_1 emergence, causing low seed damage and weight loss as well as reducing the high residual toxicity thirty, sixty and ninety days after treatment. This is followed by the petroleum oil extract of *A. boonei* stembark, while the least effective was the acetone oil extract of *A. boonei* stembark. Significantly, less eggs were laid, at all of the tested concentrations by the bruchid on the cowpea seeds protected with the *A. boonei* stembark oils extracted with five solvents compared with the numbers of eggs laid on the untreated cowpea seeds. Previous studies have reported the insecticidal activity of *A. boonei* after four days of treatment (Ileke and Oni 2011; Ileke *et al*., 2012; Ileke *et al*., 2013; 2014). Ileke and Oni (2011) reported the insecticidal potential of *A. boonei* stembark powder after four days of treatment against *Sitophilus zeamais*. Ileke *et al*. (2012 and 2013) reported the insecticidal activity of *A. boonei* powder and latex after four days of treatment against *C. maculatus* and the response of cowpea bruchid to the treatment with a 2% of *A. boonei* stembark oils extracted with methanol, ethanol, acetone, petroleum ether, and n-hexane using cold extraction methods. Ileke *et al*. (2014) reported the insecticidal activity of *A. boonei* latex after four days of treatment against *C. maculatus*. The present study confirmed the earlier reports of the insecticidal potential of *A. boonei* stembark oils and the persistence of bioactive compounds present in the studied plant part. The oils were able to protect the seeds up to three months after treatment. The plant extracts contain some chemical compounds of the triterpenoids, indole and alkaloid groups such as alstonine, astondine, and porphine (Phillipson *et al*. 1987).

The greater effectiveness of n-hexane, petroleum ether (non-polar) oils over the methanol, ethanol (polar) oils may be a result of the more bioactive compounds in the non-polar oils than the polar oils Ho *et al*. (1994, 1995, 1996). The undamaged cowpea seeds treated with non-polar and polar oils of *A boonei* stembark may be attributed to the oil content of the plant part, which could have blocked the respiratory tracts (spiracles) of the insects, leading to their death and also reducing the F_1 generation and the seed damage (Dike and Mbah, 1992; Akinkurolere, 2012). The non-effectiveness of acetone oils compared to the non-polar and polar oils may be ascribed to the polarity of acetone which is intermediate between polar (methanol, ethanol) and non-polar (n-hexane, petroleum ether) solvents, which means it may not be able to extract all the polar or the non–polar constituents of the powdered *A. boonei* stembark. Okosun and Adedire (2010; 2017) reported the non-effectiveness of the acetone extract of *Monodora myristica* seeds against *C. maculatus*. Su (1989) reported a lesser toxicity of the acetone extract of *Myristica fragnans* to *C. maculatus*, *Lasioderma serricorne* and *T. castaneum*, though it was found moderately toxic to *Sitophilus oryzae*.

The *A. boonei* stembark oils did not completely prevent oviposition by *C. maculatus* on cowpea seeds. Nevertheless, the results indicate that *A. boonei* stembark oils manifested great anti-oviposition activity against the *C. maculatus* based on the insignificant percentage of adult emergence. At higher concentrations of 3 % and 4 %, the *A. boonei* stembark oils made the cowpea seeds immune to *C. maculatus* attacks even after three months of treatment. The oils may prevent the bruchids from moving freely thereby preventing mating among adult insects (Wolfson *et al*., 1991). The inability of the insect to oviposit resulted in insignificant weight and damage losses. The perforation index was also minimal compared with the negative protectant (above 50%) recommended by Fatope et al. (1995).

5. Conclusion

The novelties in the use of *A. boonei* stembark oils extracted with five solvents (methanol, ethanol, acetone, Petroleum ether, and n-hexane) using soxhlet extraction method as long-term storage protectants (30, 60 and 90 days) against *C. maculatus* have been highlighted in this study. The *Alstonia boonei* stembark oil extracted with non-polar and polar solvents could serve as biopesticides for the protection of cowpea seeds against infestation by cowpea bruchid, *C maculatus*. The anti-oviposition exhibited by the studied plant part was greatly reflected in the beetle perforation index which is insignificant compared with negative protectants (above 50%) recommended by Fatope *et al*. (1995). The plant is eco-friendly, biodegradable and readily available in the tropical region. The oils can be ranked in terms of their effectiveness as follows: n-hexane > Petroleum ether > ethanol > methanol > acetone.

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References

Abbott WS. 1925. A method of computing the effectiveness of an insecticide. *J. Econom. Entomol.*, **18**: 265-267.

Abel C and Busia K. 2005. An Exploratory Ethnobotanical Study of the Practice of Herbal Medicine by the Akan Peoples of Ghana (Ethnobotanical Study: Herbal Medicine in Ghana). *Alternat Med Rev.,* **42**.

Adedire CO and Lajide L. 1999. Toxicity and oviposition deterrency of some plants extracts on cowpea storage bruchid, *Callosobruchus maculatus* Fabricius. *J Pl Dis Protect.,* **106**: 647 - 653

Akinkurolere RO. 2007. Assessment of the insecticidal properties of *Anchomanes difformis* (P. Beauv.) powder on five beetles of stored produce. *J Entomol*., **4**: 51-55.

Akinkurolere RO. 2012. Comparative effects of three plant powders and pirimiphos-methyl against the infestation of *Callosobruchus maculatus* (F.) (Coleoptera: Bruchidae) in cowpea seeds. *SOAJ Entomol. Stud*., **1**: 87 – 99

Akinkurolere RO, Adedire CO and Odeyemi OO. 2006. Laboratory evaluation of the toxic properties of forest anchomanes, *Anchomanes difformis* against pulse beetle *Callosobruchus maculatus* (Coleoptera: Bruchidae). *Insect Sci*., **13**: 25-29.

Ashamo MO and Akinnawonu O. 2012. Insecticidal efficacy of some plant powders and extracts against the Angoumois moth, *Sitotroga cerealella* (Olivier) [Lepidoptera: Gelechiidae]. *Arch. Phytopath. Crop Protect.,* **45**(9):1051 - 1058

Betti JL. 2004. An ethnobotanical study of medicinal plants among the Baka Pygmies in the dja biosphere reserve, Cameroon. *African St. Monog.,* **25**(1): 1 - 27.

Boeke SJ, Van Loon JJA, Van Huis A, Kossou DK and Dick M. 2001. **The Use of Plant Materials to Protect Leguminous Seeds against Beetles**. A Review: Backhuys Publishers, the Netherland, 108pp.

Dike MC and Mbah OI. 1992. Evaluation of the lemon grass products in the control of *Callosobruchus maculatus* on stored cowpea. *Nigerian J. Crop Protect.,* **14**: 88 – 91

Fatope MO, Mann A and Takeda Y. 1995. Cowpea weevil bioassay: A simple prescreen for plants with grain protectant effects. *Internat. J Pest Manag.,* **41**: 44 - 86.

Ho SH, Cheng LPL, Sim KY and Tan HTW. 1994. Potential of cloves (*Syzygium aromaticum* (L.) Merr. And Perry) as a grain protectant against *Tribolium castaneum* and *Sitophilus zeamais. Postharv. Biol Technol.,* **4**: 179 – 183.

Ho SH, Ma Y, Goh PM and Sim KY. 1995. Star anise *Illicium verum* as a potential grain protectant against *Tribolium castaneum* and *Sitophilus zeamais. Postharv. Biol Technol.,* **6**: 341 - 347

Ho SH, Koh L, Ma Y, Huang Y and Sim KY. 1996. The oil of Garlic, *Allium sativum* L. as a potential grain protectant against *Tribolium castaneum* and *Sitophilus zeamais. Postharv. Biol Technol.* **9**: 41 – 48.

Idoko JE. 2016. Evaluation of Some Paddy Rice Products as Anti-Ovipositant against *Callosobruchus maculatus* Fabricius. (Coleoptera: Chrysomelidae), *Appl Trop Agricul.,* **21**(1): 133-137

Idoko JE and Adesina JM. 2012. Evaluation of the powder of *Piper guineense* and Pirimiphos methyl for the control of cowpea beetle *Callosobruchus maculatus* (F.). *J Agricul Technol*., **8**(4): 1365 - 1374.

Ileke KD, 2014. **Insecticidal and toxicological studies on *Alstonia boonei* De Wild used as cowpea protectant against *Collosobruchus maculatus* (Fab.)**. Ph.D Thesis, Federal University of Technology, Akure, Nigeria. 269p.

Ileke KD and Oni MO. 2011. Toxicity of some plant powders to maize weevil, *Sitophilus zeamais* (Coleoptera: Curculionidae) on stored wheat grains. *African J Agricul Res.,* **6**(13): 3043 - 3048.

Ileke KD, Odeyemi OO and Ashamo MO. 2012. Insecticidal activity of *Alstonia boonei* De Wild powder against cowpea bruchid, *Callosobruchus maculatus* (Fab.) [Coleoptera: Chrysomelidae] in stored cowpea seeds. *Internat J Biol.,* **4**(2): 125 – 131

Ileke KD, Odeyemi OO, Ashamo MO, 2013. Response of cowpea bruchid, *Callosobruchus maculatus* (Fabr.) [Coleoptera: Chrysomelidae] to cheese wood, *Alstonia boonei* De Wild stembark oil extracted with different solvents. *Arch Phytopathol. Crop Protect.,* **46**(11): 1357 – 1370

Ileke KD, Oni MO and Adelegan OA. 2014. Laboratory evaluation of some plants latex as biopesticide against cowpea bruchid, *Callosobruchus maculatus* (Fab.) [Coleoptera: Chrysomelidae]. *J Agricul Sci.,* **6**(1): 123 – 128

Ileke KD, Adesina JM and Obajulaye EO. 2016. Synergetic effects of two botanicals entomocides as pest-protectants in maize grains. *J Biol Res.,* **89** (2): 33 - 39.

Lale NES and Abdulrahman HT, 1999. Evaluation of neem (*Azadirachta indica* A. Juss.) seed oil obtained by different methods and neem powder for the management of *Callosobruchus maculatus* (F.) (Coleoptera: Bruchidae) in stored cowpea. *J Stored Prod Res.,* **35**: 135 - 143.

Moronkola DO and Kunle OF. 2012. Essential Oil Compositions of Leaf, Stembark rnd Root of *Alstonia boonei* De Wild (Apocyanaceae). *Inter J Biol Pharmaceut Res.,* **3**(1): 51 - 60.

Odeyemi O O and Daramola A M. 2000. **Storage Practices in the Tropics: Food Storage and Pest Problems.** First Edition, Dave Collins Publication, Nigeria Vol. 1. 235pp.

Odeyemi OO, Gbaye OA and Akeju O. 2006. **Resistance of *Callosobruchus maculatus* (Fab.) to Pirimiphos methyl in Three Zones in Nigeria**. *Proceedings of the 9th International Working Conference on Stored Product Protection, Brazil*, pp 324 - 329.

Ofuya TI. 2001. Biology, ecology and control of insect pests of stored pulses in Nigeria. *In:* Ofuya, TI and Lale NES. (Eds), **Pest of Stored Cereals and Pulses in Nigeria: Biology, Ecology and Control**. Dave Collins Publication, Akure, Nigeria. pp 24-58

Ogunwolu EO and Odunlami AT. 1996. Suppression of seed bruchid *Callosobruchus maculatus* development and damage on cowpea (*Vigna unguiculata* (L.) Walp.) with *Zanthoxylum zanthoxyloides* (Lam). Waterm. (Rutaceae) root back powder when compared to neem seed powder and Pirimiphos-methyl. *Crop Protect.*. **15**: 603 - 607.

Okonkwo EU and Okoye WI. 1996. The efficacy of four seed powders and the essential oils as protestants of cowpea and maize grains against infestation by *Callosobruchus maculatus* and *Sitophilus zeamais. Nigerian Inter J Pest Manag.*, **42**(3): 143 - 146

Okosun OO and Adedire CO. 2010. Potency to cowpea seed bruchid, *Callosobruchus maculatus of* African Nutmeg seed, *Monodora myristica* extracted with different solvents. *Nigerian J Entomol.*, **27**: 89 – 95.

Okosun OO and Adedire CO. 2017. Insecticidal Activities of African Nutmeg Solvent Extracts Agains tCowpea Seed Bruchid, *Callosobruchus maculatus* (Fabricius) (Coleoptera: Bruchidae). *Asian J Agricul. Res.,* **11**(3): 86 – 92

Park C, Kim S and Ahn YJ. 2003. Insecticidal activity of asarones identified in *Acorus gramineus* rhizome against three coleopteran stored-product insects. *J Stored Prod Res.,* **39**: 333 - 342.

Phillipson JD, O' Neill MJ, Wright CW, Bray DH and Warhaurst DC, 1987. **Plants as a Sources of Antimalarial and Amoebicidal Compound; Medicinal and Poisonous Plants of the Tropics**: Proceedings of symposium 5 – 35 of the 14th International Botanical Congress Berlin. pp 70 – 78.

Singh SR. 1985. Insects damaging cowpeas in Asia. *In*: Singh S.R., Rachie K.O. (Eds.) **Cowpea Research, Production and Utilization**. John Wiley and Sons, Chichester, pp. 245 - 248.

Singh SR and Jackai LEN, 1985. Insect pests of cowpeas in Africa: their life cycle, economic importance and potential for control. *In*: Singh S.R., Rachie K.O. (Eds.) **Cowpea Research, Production and Utilization**. John Wiley and Sons, Chichester, pp. 217-232.

Southgate BJ. 1978. The importance of the bruchidae as pests of grain legumes, their distribution and control. *In*: Singh S.R., van Emden H.F., Taylor T.A. (Eds.), **Pests of Grain Legumes: Ecology and Control**. Academic Press, London, pp. 219-229.

Su HCF. 1989. Effect of Myristica fragrans fruit to four species of stored products. *J Entomol., Sci.* **24**: 317 – 320

Tapondjoun LA, Alder A, Bonda H and Fontem DA. 2002. Efficacy of powder and oil from *Chenpodium ambrosioides* leaves as post-harvest grain protectants against six stored products beetles. *J Stored Prod Res.,* **38**: 395 - 402.

Terashima H. 2003. Names, use and attributes of plants and animals among the Ituri forest foragers: a comparative ethnobotanical and ethnozoological study. *Afr Stud Monogr.,* **28**(Suppl.): 7 - 24.

Wolfson JL, Shade RE, Mentser PE and Murdock LL. 1991. Efficacy of ash for controlling infestation of *Callosobruhus maculatus* (F) (Coleoptera: Bruchidae) in stored cowpeas. *J Stored Prod Res.,* **27**: 239 – 243.

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Molecular Characterization of Microbial Community Diversity Associated with Blood Cockles (*Anadara granosa*) in Blood Cockle Farms

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Abstract

Microbial communities of blood cockles (*Anadara granosa*) collected from blood cockle farms at the coastal area of Northern Malaysia were examined using a standard cultivation method and 16S rRNA Illumina sequencing analysis. This study is aimed at identifying the major abundance of blood cockles' microbes and their potential relationship with different farm locations and environmental conditions. 16S rRNA Illumina sequencing and culturable microbial numbers were found to be slightly different among the samples in two different farms probably because of some environmental factors such as pollution, fresh water load, and the distance or closeness between the farm location and agricultural and industrial zones. The results indicated that most of the microbes found were typically present in blood cockles and in highly dynamic communities. The results revealed that there were slight similarities among the sampling times, and significant differences regarding the microbes' numbers between the different farm locations. Based on these results, the blood cockle microbial communities were highly dynamic and were greatly predominant by *Vibrio* spp., *Klebsiella* spp. and *Bacillus* spp. Other microbial genera found were *E. coli*, *Aliivibrio* spp., *Pseudomonas* spp., *Staphylococcus* spp., *Pseudoalteromonas* spp., and *Micrococcus* spp. existing in a minor abundance. These findings demonstrate the microbial diversity and the major abundant species in blood cockles. Hopefully, this study provides a good understanding of blood cockle microbial communities.

Keywords: Blood cockle, Environment, Coastal area, 16S rRNA genes, Sequencing.

1. Introduction

Microbial communities in marine and estuarine environments are diverse and dynamic. Some microbes can be easily found in marine and estuarine environments including *Vibrio* spp., *Pseudomonas* spp., *Klebsiella* spp., *Bacillus* spp., *Micrococcus* spp., *Photobacterium* spp., and *Aliivibrio* spp. (Khan Chowdhury *et al.*, 2009; Woodring *et al.*, 2012; Mala *et al.*, 2014; Zarkasi *et al.*, 2017a; Zarkasi *et al.*, 2017b; Zarkasi and Nazari, 2018; Lau *et al.*, 2019). Allochthonous is the most common microbial species associated with blood cockles, since blood cockles consume the surrounding water and are directly exposed to the marine environments, where those microorganisms are present (Khan Chowdhury *et al.*, 2009; Sutthirak and Boonprasop, 2011; Woodring *et al.*, 2012; Ghaderpour *et al.*, 2014; Zarkasi *et al.*, 2017a). Therefore, the presence of pathogenic microorganisms in blood cockles has become a major concern because they may put the consumer's health at risk and jeopardize farmers and fishermen's sources of income. Such pathogenic microorganisms in blood cockles can also serve as an indicator for faecal pollutions for the respective locations.

Contamination with certain microbes such as *Vibrio vulnificus*, *E. coli* and *Vibrio parahaemolyticus* may cause blood cockle illnesses as reported by other studies (Sarkar *et al.*, 1987; Khan Chowdhury *et al.*, 2009; Woodring *et al.*, 2012; Mala *et al.*, 2014). Human infections with *V. parahaemolyticus* are usually linked to improper food handling or raw seafood consumption (Johnson *et al.*, 1984; Robert-Pillot *et al.*, 2014). Moreover, *V. parahaemolyticus* is an important causative agent of gastroenteritis in humans. In addition, elements of weather and climate (Khan Chowdhury *et al.*, 2009), pollution (DePaola *et al.*, 1990), faecal pollution, storage, handling and management practices (Khan Chowdhury *et al.*, 2009) can also determine the variability of incidence and distribution of food poisoning/illnesses associated with the blood cockles in spite of the fact that most strains of seafood and environmental isolates are likely to be virulent (Khan Chowdhury *et al.*, 2009; Norhana *et al.*, 2016).

Nowadays, major blood cockle farms are located near agricultural lands, or close to residential and industrial areas. Previous studies have found out that blood cockle microbial communities are highly dynamic and sensitive to environmental factors and management practices (Khan Chowdhury *et al.*, 2009; Sutthirak and Boonprasop, 2011;

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Woodring *et al.*, 2012). Therefore, studying and understanding blood cockle microbiota and their influences can potentially lead to the protection of the environment, as well as the improvement of storage, transportation, and management practices of blood cockle products which may eventually contribute to blood cockle farming and help boost industrial sustainability. The aim of this study is to identify the microbial communities associated with blood cockles (*Anadara granosa*) and to study the potential factors that may influence these microbial communities. The primary questions which the current study attempts to answer are: what are the most abundant microbes associated with blood cockles (*Anadara granosa*), and whether the sampling location and the surrounding environments affect blood cockles' microbial communities or not?

2. Materials and Methods

2.1. Sample Collection

Blood cockle samples were collected from blood cockle farms located at coastal area in Juru and Kerian, Malaysia in August and October, 2017. Juru area is located near to an industrial zone, and is already known for its big pollution problem (Yap *et al.*, 2008), while Kerian area is located near an agriculture zone (paddy field) and a residential area. The samples collected from Juru are referred to as 'JU', while samples collected from Kerian are referred to as 'KR'. A total of twenty-four samples (twelve samples per sampling location) were randomly collected. They were, then, immediately transported in a chilling ice box to the laboratory and were processed within three hours.

2.2. Microbial Enumeration and Cultivation

Based on the sampling location, the blood cockle samples were divided into two different groups (JU and KR). The blood cockle samples were then examined thoroughly, and their colour, smell, and gross appearance were all recorded. The samples were then cleaned with a brush under running tap water to remove any sand, debris or mud on their shells. After that, the raw blood cockles were aseptically shucked, and the intact bodies and liquor were placed and pooled into a sterilized filter blender bag. The bag was massaged through by hand for one minute to separate the excess shell from the liquor and intact bodies. In a process to remove the remaining shells, the samples were then transferred into a new full filter blender bag, to which 450 ml of 3 % sea salt peptone water was added. The samples were allowed to homogenize for two minutes (Zarkasi and Nazari, 2018). Samples of 5 ml were taken and processed for microbial enumeration and DNA extraction respectively. Then, serial dilutions were performed and spread onto three different types of agar media, namely Marine Agar (MA), Brain-Heart Infusion (BHI) Agar with 3 % sea salt and thiosulfate-citrate-bile salts-sucrose (TCBS) agar (Zarkasi and Nazari, 2018). After that, the plates were incubated at 25ºC for 24-72 hours according to aerobic and anaerobic atmospheres (AnaeroGen kit by Oxoid). All plates were examined by the standard plate count method after 24-72 hours of incubation. One loop of the suspected growing colony was taken and streaked onto a Nutrient Agar (NA) media to get a pure colony for characterization and identification. In total, thirty-one colonies were chosen for identification.

2.3. DNA Extraction and 16S rRNA Gene Analysis for Pure Colonies

Using Eppendorf tubes containing sterile distilled water, a single colony from a pure culture was transferred into the tube and heated to 70°C for ten minutes, and was then centrifuged (4000 x g, 1 min). PCR was then performed using 2 µL of the heated extract with final concentrations of the PCR reaction mix including 1 µL(20 pmol) of each of primers 341F (5' CTA CGG GAG GCA GCA G) and 907R primer (5' AAA CTC AAA GGA ATT GAC) (GeneWorks, Australia), 1 µL of bovine serum albumin, 12.5 µL of ImmoMix (Bioline, UK), and 7.5 µL of sterile distilled water to a final volume of 25 µL. Thermocycling was performed using a C1000 Thermal Cycle (Bio-Rad, California, US) at 95°C for ten minutes, 94°C for one minute, 55°C for one minute, 72°C for one minute; repeated for twenty-three cycles,72°C for ten minutes, and soaked at 15°C (Zarkasi *et al.*, 2014; Zarkasi and Nazari, 2018). The purified amplicons were then sequenced using an ABI 3730 automated sequencer using the Big Dye direct cycle sequencing kit. A comparison of individual rRNA gene sequences to those published in the BLAST database (http://blast.ncbi.nlm.nih.gov/) was done to determine the microbial genera (Neuman *et al.*, 2016).

2.4. Direct Total DNA Extraction

The total microbial DNA was extracted directly from the twenty-four blood cockle samples using the QIAamp DNA Mini Kit (QIAGEN Sciences, Germantown, MD, US) following the manufacturer's instructions. The direct DNA extraction was performed soon after sampling or on samples that were maintained frozen at temperature -80°C.

2.5. 16S rRNA gene Sequencing Using Illumina MiSeq Platform

The sequencing of the 16S rRNA gene amplicon was applied to the twenty-four samples collected from blood cockle farms, to examine the microbial communities and diversity present in each of the samples. Sequencing was carried out using the Illumina MiSeq platform (Zarkasi *et al.*, 2018). Pair-ended PCR amplification of the 16S rRNA gene V3-V4 region was carried out using 341F and 907R primers. The FASTQ files generated were merged using PEAR (Zhang *et al.*, 2012), and these were then trimmed to remove the primer, barcode, and adapter regions. The seed sequence for each cluster was then sorted by length and clustered with a 4 % divergence cut-off to create centroid clusters. The clusters containing only <2 sequences or <100 bp in length were then removed. The seed sequences were again clustered at a 4 % divergence level using USearch to confirm whether any additional clusters appeared. Consensus sequences from these clusters were then accurately obtained using UPARSE (Edgar, 2013). Each consensus sequence and its clustered centroid of reads was then analyzed to remove chimeras utilizing UCHIME in the *de novo* mode (Edgar *et al.*, 2011). After chimera removal, each consensus sequence and its centroid cluster were denoised in UCHIME in which the base position quality scores of >30 acted as the denoising criterion. Sequence de-replication and OTU demarcation was further performed in USEARCH and UPARSE to yield OTUs that were aligned using MUSCLE

(Edgar, 2004) and FastTree (Price *et al.*, 2010) which infers approximate maximum likelihood phylogenetic trees. OTUs were then classified using the RDP Classifier (Wang *et al.*, 2007) against the curated GreenGenes 16S rRNA gene database (DeSantis *et al.*, 2006; Hussin *et al.*, 2018).

2.6. Statistical Analysis

PRIMER6 and PERMANOVA+ (Primer-E, Ivybridge, UK) respectively were used to conduct analysis of variance (ANOVA) and Multidimensional scaling (MDS) to assess the influence of different factors on community compositions. The ANOVA-derived significance values were considered significant when $P < 0.01$, while $0.01 < P < 0.05$ were considered marginally significant (Zarkasi *et al.*, 2016).

3. Results

3.1. Culturable Microbial Population Structure

The water surface (5 m) temperature recorded during sampling ranged between 27 and 29°C. The results from culturable plates show that the average viable counts of JU and KR collected from MA, BHI and TCBS were varied. For JU, the average viable counts were 7.17 log cfu/g on MA, 7.13 log cfu/g on BHI and 7.56 log cfu/g on TCBS, while the KR samples average viable counts were 4.66 log cfu/g on MA, 4.76 log cfu/g on BHI and 4.53 log cfu/g on TCBS (Figure 1). However, the following month, the average viable counts were almost the same compared with the previous month. The average viable counts for JU were 7.20 log cfu/g on MA, 7.19 log cfu/g on BHI and 7.50 log cfu/g on TCBS, while for KR they were 4.69 log cfu/g on MA, 4.78 log cfu/g on BHI and 4.49 log cfu/g on TCBS (Figure 1).

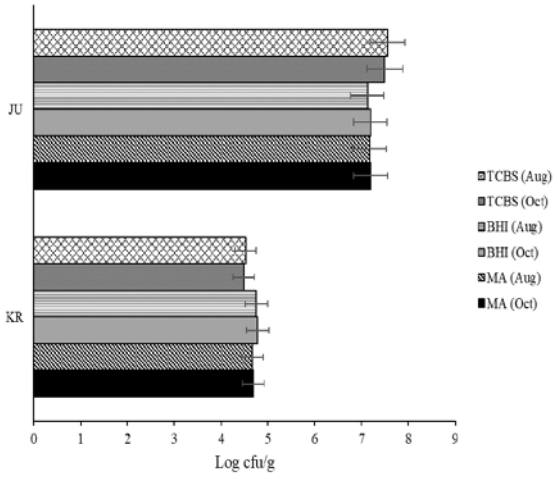

Figure 1. Total viable counts (TVC) populations derived from the colony numbers appearing on marine agar, BHI agar and TCBS agar, for bacterial cultured from blood cockle (*Anadara granosa*) according to the location of sampling. The bars equal to the Standard Error (SE).

3.2. Microbial Communities of Blood Cockles are Dominated by Members of the Family Vibrionaceae and Enterobacteriaceae

The major dominant microbes described from this study based on 16S rRNA Illumina gene analysis were bacterial groups belonging to the family of Vibrionaceae (*Vibrio, Aliivibrio* and *Photobacterium*) making up >36 % of the total numbers. These were followed by Enterobacteriaceae (*Escherichia, Klebsiella* and *Citrobacter*) making up >32 % of the total numbers, and also Bacillaceae (*Bacillus* and *Geobacillus*), which constituted >19 % of the total numbers (Figure 2).

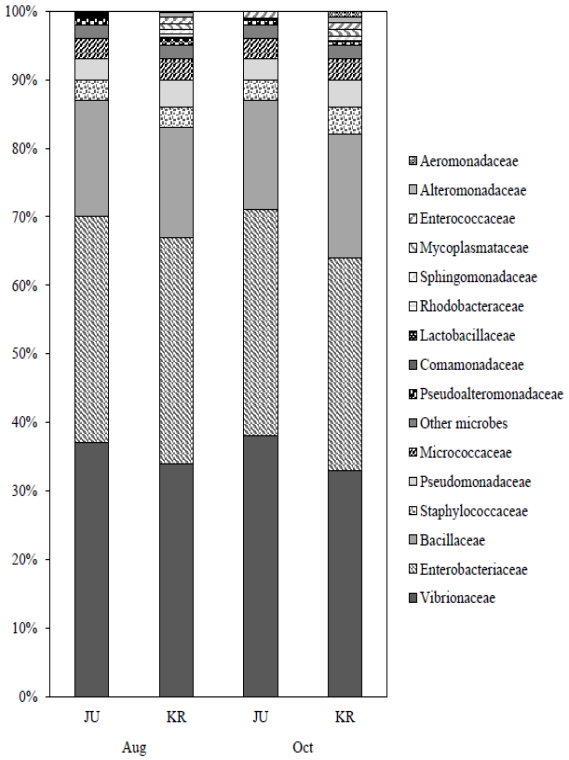

Figure 2. Relative abundances (in % of total numbers) of the most abundant microorganisms at family level associated with blood cockle.

The results show consistency among the JU and KR samples indicating its population dynamics. Other microbe families such as Staphylococcaceae, Pseudomonadaceae, Aeromonadaceae, Alteromonadaceae, Enterococcaceae, Mycoplasmataceae, Sphingomonadaceae, Rhodobacteraceae, Lactobacillaceae, Comamonadaceae, Pseudoalteromonadaceae, and Micrococcaceae were also identified making up >13 % of the total numbers (Figure 2). Down to the genera level, the most abundant microbial genera were *Vibrio* spp., *Klebsiella* spp., and *Bacillus* spp. making up ~28 %, ~20 % and ~16 % of the total number respectively (Figure 3). Besides, *E. coli* (~15 % of the total number), *Staphylococcus* spp. (~4 % of the total number), *Micrococcus* spp. (~3 % of the total number), *Pseudomonas* spp. (~3 % of the total number), and *Pseudoalteromonas* spp. (~2 % of the total number) were also significantly present as visualised by the heat map (Figure 3). Other microbial genera such as *Sphingomonas, Paracoccus, Enterococcus, Lactobacillus, Chryseobacterium, Flavobacterium, Citrobacter, Enterobacter* and *Streptococcus, Clostridium, Geobacillus,* and *Aeromonas* were also identified making up >7 % of the total numbers

(Figure 3). An interesting observation was the significant number of coliform bacterial reads (~15 % of reads on average (Figure 3). These reads were dominated by the *Escherichia coli*, the most popular coliform bacteria.

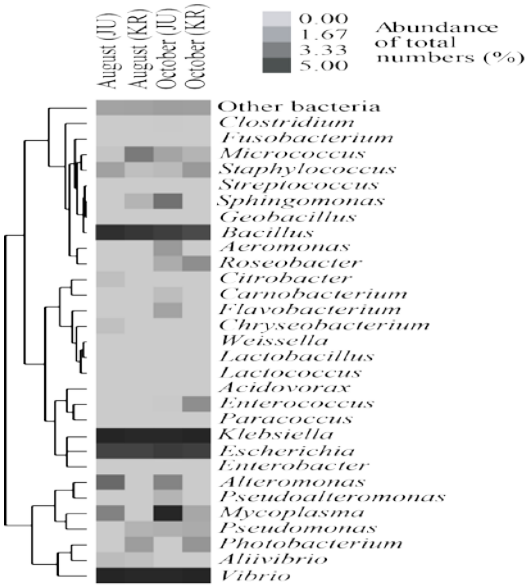

Figure 3. Heat map and hierarchical clustering plot of the blood cockle bacterial communities identified via 16S rRNA Illumina gene analysis.

3.3. Microbial Diversity

The distribution and diversity of microbial community structure associated with blood cockles in the different locations were slightly different, and there was a small clear separation as seen through the MDS plots based on 16S rRNA Illumina gene data (Figure 4). This is supported by the analysis of variance (ANOVA) results which indicated that the sampling locations were slightly different ($0.01 < P < 0.05$). However, as for the sampling time, there was no significant difference ($P > 0.05$). Further analysis using pairwise tests showed that the populations varied and were marginally significant ($0.01 < P < 0.05$). Marginal separation was observed between the JU and KR ($P = 0.04$) (Figure 4).

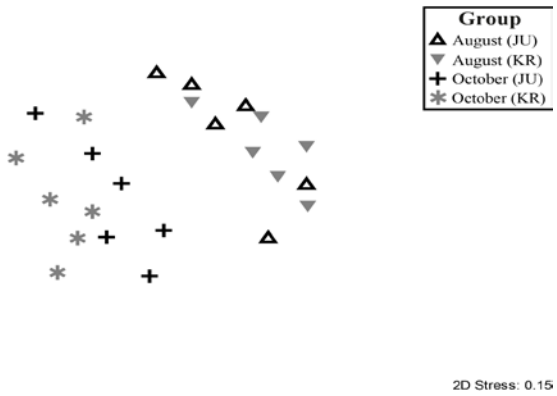

Figure 4. MDS plots showing blood cockle microbial communities.

4. Discussion

The traditional morphology, culture-dependent identification, and biochemical criteria had limitations in terms of comparisons between the isolates and non-culturable microbes because some of the microbial species are not cultivable (Hovda *et al*., 2007). Therefore, the use of a molecular approach such as the next generation sequencing especially for the microbial identification in fish and marine animals has become more important nowadays because the advancement of identification and morphology technology as well as its capability to detect uncultivable bacteria and other bacteria require special growth conditions (Tarnecki *et al.*, 2017).

The numbers of microbial genera groups present in the blood cockles (*Anadara granosa*) that were collected from blood cockle farms, located at coastal area in northern Malaysia, were investigated and analysed in this study. This study maintains that the microbial communities in the blood cockles are dynamic as explained earlier in response to some environmental factors, product handling, transportation, storage, and management practices (Khan Chowdhury *et al.*, 2009; Sutthirak and Boonprasop, 2011; Woodring *et al.*, 2012; Zarkasi and Nazari, 2018). This study shows that blood cockle bacteria were allochthonous, in which the dynamic influence of microbial communities was due to external factors (Neuman *et al.*, 2016; Zarkasi *et al.*, 2016) such as temperature, season, human or animal activity, geographical location, and pollution. Both results from cultivation and 16S rRNA Illumina gene sequencing produce almost similar results. However, 16S rRNA Illumina gene sequencing data provide more details of microbial communities since 16S rRNA Illumina gene sequencing can identify non-cultivation microbes. This is the benefit of the application of 16S rRNA Illumina gene sequencing techniques in the study of microbial ecology to get a deeper understanding of microbial communities (Zarkasi *et al.*, 2018).

The environmental factors and management practices in blood cockle farms, transportation, handling and storage may influence the microbial communities and its population dynamics. This indication is supported by the high numbers of *Vibrio* spp., *Klebsiella* spp. and *Bacillus* spp. (Figure 3). The significant difference between microbial communities from different farms indicates that environmental factors do affect the microbial communities associated (Mohamad Suhaimi *et al.*, 2019) with blood cockles. The JU farm was located near an industrial estate, while the KR farm was located near an agricultural (paddy) and residential area. That explains why the microbial communities in the two farms were distinct, since the JU farm location was reported by previous studies to be polluted by the industrial activities (Yap *et al.*, 2008). Leavitt, (2009) explains how microbial contamination in the coastal area could have been caused by environmental factors such as pollution, or being located close to a residential, industrial, or farming area (Heath *et al.*, 1995). According to some researchers, these bacteria were easily found in blood cockles (Areerat, 2000).

The presence of *E. coli*, *Vibrio* spp., and *Staphylococcus* spp. may have been caused by environmental factors in blood cockle farms since the farms are located at coastal estuaries. Moreover, the coastal areas in northern Malaysia are known to be exposed to pollution coming from the industrial zone, residential areas, or agricultural farms (paddy field) nearby (Yap *et al.*, 2008; Khan Chowdhury *et al.*, 2009; Sutthirak and Boonprasop, 2011; Woodring *et al.*, 2012; Ghaderpour

et al., 2014; Shahunthala, 2015). Those bacterial genera detected by this study were also found in other marine organism (Hovda *et al.*, 2007; Zarkasi *et al.*, 2016) , and these bacteria are well-known to cause food spoilage and food-borne illnesses to the consumers. This study recommends that any research conducted on pollution must also discuss microbial communities present at the targeted locations in addition to analyzing the effect of chemical compounds. Frequent incidences were reported across the globe and raised concerns regarding blood cockle consumption (Urbanczyk *et al.*, 2007).

The bacterial genera of *Vibrio* spp., and *Escherichia coli* in aquaculture are important because they are considered as an indicator for faecal pollution and can cause food-borne illnesses to blood cockle consumers (Eng *et al.*, 1989; Austin, 2006; Austin and Zhang, 2006; Norhana *et al.*, 2016). However, disease incidence depends on the number of bacteria present and whether it possesses specific virulence determinants (Cao *et al.*, 2009). The interesting part in this study is the presence of *Pseudoalteromonas* spp. (Zarkasi and Nazari 2018) because this bacterium is known to be found in spoiled blood cockles. This provides an answer to the question why some blood cockle products have a shorter lifespan, which is attributed to the presence of these bacteria inside the blood cockles (Norhana *et al.*, 2016). The dynamism in the blood cockle microbial community structure is more interesting due to the significant level of coliform bacteria presence, despite the presence of high levels of Vibrionaceae. Coliform bacteria predominant in the samples as in the JU farm, show the substantial variation between individual blood cockles and potentially reflect stochastic exposure patterns between individuals, and environmental influences.

This study shows distinct results from different farms, and the morphological and 16S rRNA Illumina analysis results provide almost identical results, which gives a conclusive evidence of the microbial community dynamics in the blood cockles by this study. The dominant bacteria identified can be considered typical to marine organisms including blood cockles (Mala *et al.*, 2014; Zarkasi and Nazari, 2018). Based on the results (Figure 4), the microbial community from blood cockles reflects and is influenced by the surrounding environments and the location of the sampling site (Hatje *et al.*, 2014). The sampling location in the JU area was close to an industrial zone, while the KR farm was located close to an agricultural and residential area. Moreover, according to Llewellyn *et al.*, (2016) and Sullam *et al.*, (2012), the microbial communities in marine animals such as fish and blood cockles are influenced and shaped by the surrounding environments and geographical locations. Furthermore, the blood cockles collected from blood cockle farms at coastal/estuaries in northern Malaysia are possibly polluted by the surrounding environment (Ghaderpour *et al.*, 2014; Khodami *et al.*, 2017); according to this study, the microbial diversity in blood cockles is influenced by location and environmental factors. Similarly, previous also maintained that the surrounding environment can influence the microbial diversity of blood cockles, oyster and fish through many factors such as seawater temperature, geographical location, seasonal period, pollution, and farming techniques (Zarkasi *et al.*, 2014). Identifying this bacterium as part of the blood cockles can be of interest to determine contamination routes and study possible pollution from the surrounding environments.

5. Conclusion

This study concludes that *Vibrio* spp., *Klebsiella* spp., and *Bacillus* spp. are the most predominant bacterial genera associated with blood cockles (*Anadara granosa*), while *E. coli*, *Pseudomonas* spp., *Aliivibrio* spp., *Staphylococcus* spp., *Pseudoalteromonas* spp., and *Micrococcus* spp. are also commonly abundant. The findings of this study confirm that these bacteria are typically isolated from blood cockles and other marine animals with highly dynamic microbial communities (Yap *et al.*, 2008; Khan Chowdhury *et al.*, 2009; Woodring *et al.*, 2012; Ghaderpour *et al.*, 2014) and their presence raise real concerns. The results obtained could be used to improve choices regarding blood cockle farming locations, product storage and handling procedures by retailers and distributors for the sake of maintaining blood cockles' quality before reaching consumers and also improving seafood safety. This study could also be useful for understanding how environmental conditions can affect blood cockle farms. Further studies of this nature could reveal important links between blood cockle farming, environmental factors, and husbandry strategies.

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References

Areerat S. 2000. Reduction of Contaminant Bacteria in Live Blood Clam (*Anadara granosa*). Thai National AGRIS Centre, Bangkok.

Austin B. 2006. The bacterial microflora of fish, revised. *Sci World J*, **6**: 931-945.

Austin B and Zhang XH. 2006. *Vibrio harveyi*: a significant pathogen of marine vertebrates and invertebrates. *Lett Appl Microbiol*, **43**: 119-124.

Cao R, Xue CH, Liu Q and Xue Y. 2009. Microbiological, chemical, and sensory assessment of Pacific Oysters (*Crassostrea gigas*)stored at different temperatures.*Czech J Food Sci*, **27**:102-108.

DePaola A, Hopkins L, Peeler J, Wentz B and McPhearson R. 1990. Incidence of *Vibrio parahaemolyticus* in US coastal waters and oysters. *Appl Environ Microbiol*, **56**: 2299-2302.

DeSantis TZ, Hugenholtz P, Larsen N, Rojas M, Brodie EL, Keller K, Huber T, Dalevi D, Hu P and Andersen GL. 2006. Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB. *Appl Environ Microbiol*, **72**: 5069-5072.

Edgar RC. 2004. MUSCLE: a multiple sequence alignment method with reduced time and space complexity. *BMC Bioinformatics*, **5**: 113.

Edgar RC. 2013. UPARSE: highly accurate OTU sequences from microbial amplicon reads. *Nat Methods*, **10**: 996-998.

Edgar RC, Haas BJ, Clemente JC, Quince C and Knight R. 2011. UCHIME improves sensitivity and speed of chimera detection. *Bioinformatics*, **27**: 2194-2200.

Eng CT, Paw JN and Guarin FY. 1989. The environmental impact of aquaculture and the effects of pollution on coastal aquaculture development in Southeast Asia. *Marine Poll Bull*, **20**: 335-343.

Ghaderpour A, Nasori KNM, Chew LL, Chong VC, Thong KL and Chai LC. 2014. Detection of multiple potentially pathogenic bacteria in Matang mangrove estuaries, Malaysia. *Marine Poll Bull*, **83**: 324-330.

Hatje E, Neuman C, Stevenson H, Bowman JP and Katouli M. 2014. Population Dynamics of *Vibrio* and *Pseudomonas* Species Isolated from Farmed Tasmanian Atlantic Salmon (*Salmo salar* L.): A Seasonal Study. *Microb Ecol*, **68**: 679–687.

Heath A, Cole D, Bishop D, Pfeffer A, Cooper S and Risdon P. 1995. Preliminary investigations into the aetiology and treatment of cockle, a sheep pelt defect. *Vet Parasitol*, **56**: 239-254.

Hovda MB, Lunestad BT, Fontanillas R and Rosnes JT. 2007. Molecular characterisation of the intestinal microbiota of farmed Atlantic salmon (*Salmo salar* L.). *Aquacul.*, **272**: 581-588.

Hussin NA, Zarkasi KZ and Majid AHA. 2018. Characterization of gut bacterial community associated with worker and soldier castes of *Globitermes sulphureus* Haviland (Blattodea: Termitidae) using 16S rRNA metagenomic. *J Asia Pac Entomol*, **21**: 1268-1274.

Johnson DE, Calia FM, Musher DM and Goree A. 1984. Resistance of *Vibrio vulnificus* to serum bactericidal and opsonizing factors: relation to virulence in suckling mice and humans. *J Infect Dis*, **150**: 413-418.

Khan Chowdhury AJ, Musa N, Mahfuz F, Yunus K, Omar MN, Sarker MNA and Jaswir I. 2009. Bacterial pollution in molluscs arch clam, *Orbicularia orbiculata* and blood cockle, *Anadara granosa* of Pahang estuary, Malaysia. *J Biol Sci*, **9**: 841-850.

Khodami S, Surif M, Wan Omar WM and Daryanabard R. 2017. Assessment of heavy metal pollution in surface sediments of the Bayan Lepas area, Penang, Malaysia. *Marine Poll Bull*, **114**: 615-622.

Lau N-S, Zarkasi KZ, Sah ASRM and Shu-Chien AC. 2019. Diversity and coding potential of the microbiota in the photic and aphotic zones of tropical Man-Made Lake with intensive aquaculture activities: a case study on Temengor Lake, Malaysia. *Microb Ecol*, **78**: 20-32.

Leavitt DF. 2009. Best Management Practices for the Shellfish Culture Industry in Southeastern Massachusetts. USDA Risk Management Agency, Raleigh.

Llewellyn MS, McGinnity P, Dionne M, Letourneau J, Thonier F, Carvalho GR, Creer S and Derome N. 2016. The biogeography of the Atlantic salmon (*Salmo salar*) gut microbiome. *ISME J*, **10**: 1280-1284.

Mala W, Chomvarin C, Alam M, Rashed SM, Faksri K and Angkititrakul S. 2014. Molecular analysis of *Vibrio Vulnificus* isolated from cockles and patients in Thailand. *Southeast Asian J Trop Med Public Health*, **45**: 103.

Mohamad Suhaimi NS, Yeoh SY, Nazari TF, Halim MA, Daud F, Ibrahim D and Zarkasi KZ. 2019. Shrimp gut microbial community dynamic influence by the potential of shrimp feed additives of prodigiosin – an in vitro gut model. *Malays J Microbiol*, DOI: http://dx.doi.org/10.21161/mjm.180236.

Neuman C, Hatje E, Zarkasi KZ, Smullen R, Bowman JP and Katouli M. 2016. The effect of diet and environmental temperature on the faecal microbiota of farmed Tasmanian Atlantic salmon (*Salmo salar* L.). *Aquacult Res*, **47**: 660-672.

Norhana W, Yurimoto T, Nurlemsha BI and Saadon K. 2016. Food safety aspects in blood cockles (*Tegillarca granosa*) cultured off Selangor, Peninsular Malaysia. *Malays J Sci*, **35**: 210-222.

Price MN, Dehal PS and Arkin AP. 2010. FastTree 2–approximately maximum-likelihood trees for large alignments. *PLoS One*, **5**: e9490.

Robert-Pillot A, Copin S, Himber C, Gay M and Quilici ML. 2014. Occurrence of the three major *Vibrio* species pathogenic for human in seafood products consumed in France using real-time PCR. *Int J Food Microbiol*, **189**: 75-81.

Sarkar B, Kumar RD, De S and Pal S. 1987. Hemolytic activity of and lethal toxin production by environmental strains of *Vibrio parahaemolyticus*. *Appl Environ Microbiol*, **53**: 2696-2698.

Shahunthala DR. 2015. Cockle mortality in Perak and Penang coastal locations: an assessment of water quality as a contributory factor. *Malays Fish J*, **14**: 53-58.

Sullam KE, Essinger SD, Lozupone CA, O'connor MP, Rosen GL, Knight R, Kilham SS and Russell JA. 2012. Environmental and ecological factors that shape the gut bacterial communities of fish: a meta-analysis. *Mol Ecol*, **21**: 3363-3378.

Sutthirak P and Boonprasop S. 2011. Microbiological quality of fresh cockle (*Anadara granosa*) during storage at room temperature. *Thai J Agricult Sci*, **44**: 466-470.

Tarnecki AM, Burgos FA, Ray CL and Arias CR. 2017. Fish intestinal microbiome: Diversity and symbiosis unravelled by metagenomics. *J Appl Microbiol*, **123**: 2-17.

Urbanczyk H, Ast CJ, Higgins MJ, Carson J and Dunlap PV. 2007. Reclassification of *Vibrio fischeri*, *Vibrio logei*, *Vibrio salmonicida* and *Vibrio wodanis* as *Aliivibrio fischeri* gen. nov., comb. nov., *Aliivibrio logei* comb. nov., *Aliivibrio salmonicida* comb. nov. and *Aliivibrio wodanis* comb. nov. *Int J Syst Evol Microbiol*, **57**: 2823-2829.

Wang Q, Garrity GM, Tiedje JM and Cole JR. 2007. Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. *Appl Environ Microbiol*, **73**:5261-5267.

Woodring J, Srijan A, Puripunyakom P, Oransathid W, Wongstitwilairoong B and Mason C. 2012. Prevalence and antimicrobial susceptibilities of *Vibrio*, *Salmonella*, and *Aeromonas* isolates from various uncooked seafoods in Thailand. *J Food Prot*, **75**: 41-47.

Yap C, Hatta Y, Edward F and Tan S. 2008. Comparison of heavy metal concentrations (Cd, Cu, Fe, Ni and Zn) in the shells and different soft tissues of *Anadara granosa* collected from Jeram, Kuala Juru and Kuala Kurau, Peninsular Malaysia. *Pertanika J Trop Agric Sci*, **31**: 205-215.

Zarkasi KZ, Abell GCJ, Taylor RS, Neuman C, Hatje E, Tamplin ML, Katouli M and Bowman JP. 2014. Pyrosequencing-based characterization of gastrointestinal bacteria of Atlantic salmon (*Salmo salar* L.) within a commercial mariculture system. *J Appl Microbiol*, **117**: 18-27.

Zarkasi KZ, Halim MA, Nazari TF and Daud F. 2018. Metagenomic data on *Anadara granosa* associated bacterial communities using culture dependent approaches and 16S rRNA sequencing. *Data in Brief*, **19**: 514-519.

Zarkasi KZ and Nazari TF. 2018. Molecular characterisation of microbial diversity associated with oysters within a commercial oyster farm. *Turk J Fish Aquat Sci*, **18**: 191-197.

Zarkasi KZ, Sheng KF, Muhammad NA, Nazari TF and Abdullah AAA. 2017a. Bacterial community diversity associated with blood cockle (*Anadara granosa*) in Penang, Malaysia. *Sci Bruneiana*, 16(2): **41**–47.

Zarkasi KZ, Taylor RS, Abell GCJ, Tamplin ML, Glencross BD and Bowman JP. 2016. Atlantic salmon (*Salmo salar* L.) gastrointestinal microbial community dynamics in relation to digesta properties and diet. *Microb Ecol*, **71**: 589–603.

Zarkasi KZ, Taylor RS, Glencross BD, Abell GCJ, Tamplin ML and Bowman JP. 2017b. *In vitro* characteristics of an Atlantic salmon (*Salmo salar* L.) hind gut microbial community in relation to different dietary treatments. *Res Microbiol*, **168**: 751-759.

Zhang J, Zhang Y, Liu SN, Han Y and Zhou ZJ. 2012. Modelling growth and bacteriocin production by *Pediococcus acidilactici* PA003 as a function of temperature and pH value. *Appl Biochem Biotechnol*, **166**:1388-1400.

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Exogenous Jasmonic Acid Induces Lead Stress Tolerance in Kidney Bean (*Phaseolus vulgaris* L.) by Changing Amino Acid Profile and Stimulating Antioxidant Defense System

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Abstract

In this study, the impact of different lead (Pb) concentrations (100 and 1000 µM) on growth parameters, photosynthetic pigment content, relative water content (RWC), ion homeostasis, protein and amino acid profile of kidney bean (*Phaseolus vulgaris* L.) plants is investigated. In addition, the changes in antioxidant enzyme activities, including ascorbate peroxidase (ASPX), catalase (CAT), and glutathione-S-transferase (GST) have been determined. Reduced glutathione (GSH) content was also calculated. The potential of jasmonic acid (100 µM) as a stress-signaling molecule in alleviating the drastic effects of lead stress on kidney bean plants has been evaluated. The current study found out that Pb treatment significantly (P<0.05) reduced growth, chlorophyll contents, relative water content, essential and non-essential amino acid levels, and essential ions (Mg^{2+} and Ca^{2+}). The Pb effect was directly related to the applied concentrations. The results showed that JA effectively alleviates the inhibitory effect of Pb on plant growth and chlorophyll contents, probably by reducing the Pb uptake, maintaining ion homeostasis, increasing antioxidant enzyme activity (ASPX, CAT and GST), and metal-binding molecules including GSH and amino acids such as proline and cysteine. To the researchers' knowledge, this study is the first of its kind to unravel the protective role of exogenous JA in alleviating the effects of Pb stress in *Phaseolus vulgaris* L., and the underlying mechanism for the JA-induced stress tolerance.

Keywords: Amino acids, Antioxidant enzymes, Glutathione, Growth, Ion homeostasis, Jasmonate, Kidney beans, Protein.

1. Introduction

Heavy metals constitute a major problem for plants and highly affect their metabolic activities. Lead (Pb) is a very toxic environmental pollutant (Grover *et al*., 2010; Pourrut *et al*., 2011). The toxic effect is a result of lead extracting and melting processes, the use of paints containing lead, gasoline and explosives, and Pb-enriched sewage treatment and disposal (Chany and Ryan, 1994). Pb was found to accumulate in cultivated soils close to industrial areas. It is absorbed by plants and accumulates in different organs (Arshad *et al*., 2008). Pb induces a broad range of morphological, physiological, and biochemical effects that can be toxic on living organisms (Pourrut *et al*., 2011). This metal reduces seed germination, affects plant growth, and root elongation. It also impedes seedling development and chlorophyll production (Sharma and Dubey, 2005; Maestri *et al*., 2010). In addition, Pb phytotoxicity inhibits the activities of enzymes, especially those containing sulfhydryl (-SH) groups, interfering with the mineral nutrition and water balance. The hormonal status and membrane permeability get interrupted. It also induces secondary stresses, similar to those caused by nutritional deficiencies and excessive reactive oxygen species (Krämer and Clemens, 2005; Sharma and Dubey, 2005). These disorganizations disrupt the normal physiological conditions of the plant.

To cope with Pb stress, as well as stresses caused by other metals, plants have several defense strategies that are associated with the cellular-free metal content [eg., cell wall binding, metal exclusion, chelation and sequestration (Hall, 2002)] and to the control and modulation of cellular responses [eg., repair of proteins that have been damaged by stress factors and antioxidative defenses (Hall, 2002)]. The production of chelators and the upcoming trapping of metal complexes are major factors needed to limit free metal concentrations. Glutathione (GSH) is a tripeptide produced in the cell cytoplasm and chloroplasts that scavenge $^1O_2$ and hydrogen peroxide (H_2O_2). Glutathione is oxidized to glutathione disulfide, which functions as a redox regulator and an antioxidant. GSH is a substrate for glutathione-S-transferases (GSTs), which play an important role in the detoxification of xenobiotics. GSH is also a precursor of phytochelatins, which control cellular heavy metal levels, and is involved in controlling gene expression (Sofo *et al*. 2010; Seth *et al*., 2012).

Methyl jasmonate (MeJA) and its free-acid, jasmonic acid (JA), together called jasmonates, are important cellular regulators related to diverse developmental

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activities, including seed germination, root growth, fruit ripening, and senescence (Xang and Hause, 2002, Samota *et al*. 2017). In addition, jasmonates induce defense mechanisms in plants in response to pathogen attacks, wounding, and abiotic stress factors (Javid *et al*., 2011). Jasmonic acid, when added exogenously, stimulates the expression of genes involved in GSH synthesis providing protection against oxidative stress (Xang and Oliver, 1998). Recently, Ali *et al*. (2018) observed that JA could act as a "stress-ameliorating molecule" by improving the tolerance of rapeseed plants to cadmium toxicity.

Although the role of JA in protecting plants against environmental stresses, such as drought, low temperature, salinity and cadmium toxicity, has been extensively studied (Cheong and Choi, 2003; Hassanein *et al*., 2009), Pb-related tolerance strategies in JA-treated are not yet fully- understood. The objectives of this study are to investigate the role of JA acting as a growth regulator in alleviating the harmful effects of Pb on *Phaseolus vulgaris* L. and to determine the changes in the total protein pattern, amino acid profile, antioxidant enzyme activities, GSH level and ion homeostasis in response to JA in both Pb-treated and control plants.

2. Materials and Methods

2.1. Plant Material

The kidney bean (*P. vulgaris* L.) seeds were purchased from Crop Institute, Agricultural Research Center, Giza, Egypt.

2.2. Growth Conditions and Treatments

The present study was conducted in the greenhouse of Botany Department, Faculty of Science, Ain Shams University. Healthy kidney bean (*P. vulgaris*) seeds of a matching size were chosen and surface-sterilized in 0.0 5% (w/v) sodium hypochlorite solution, and were repeatedly washed with distilled water. Ten seeds were scattered in each pot at a depth of 3 cm. Five plastic pots (25-cm deep and 40-cm diameter) were used for each treatment. Each pot contained 14 kg of a blend of clay and sand (2:1 w/w). The pots were kept in a greenhouse under normal conditions (the mean light and dark temperatures were 24°C and 12°C ± 3°C, respectively). Two-week-old seedlings were irrigated with the specific lead nitrate [$Pb(NO_3)_2$] concentrations at 70 % of the soil water-holding capacity, and two concentrations of $Pb(NO_3)_2$ were used; 100 and 1,000 µM. After two days, the plants grown on each $Pb(NO_3)_2$ concentration were separated into two groups. The first group was sprayed with 100 µM of JA in 0.01% Tween 20 and the second group was sprayed with water to serve as the control. After two days of JA treatment, growth parameters, including shoot and root lengths, mean leaf area per plant, number of leaves per plant, and fresh and dry weights (FW and DW, respectively) of shoots and roots, the relative water content (RWC), photosynthetic pigments and mineral ions, including zinc (Zn), calcium (Ca) and magnesium (Mg), were measured. In addition, changes in the antioxidant enzyme activities (ascorbate peroxidase (APX), catalase (CAT) and GST) were measured. The GSH level and amino acid and protein profiles were also determined.

2.3. Determination of the Relative Water Content (RWC)

The RWC was calculated in 2-cm fresh leaf discs, excluding the midribs. Discs were weighed quickly and immediately left to float for twenty-four hours in the dark on deionized water in Petri dishes to saturate them. The water adhering to the discs was blotted away, and the turgor mass was noted. The dry masses of the discs were determined by dehydrating them at 80°C for forty-eight hours (Fariduddin *et al*., 2009). The RWC was calculated using the formula below:

$$RWC = \frac{\text{Fresh mass} - \text{dry mass}}{\text{Turgor mass} - \text{dry mass}} \times 100$$

2.4. Photosynthetic Pigments

The concentrations of photosynthetic pigments, chlorophyll a (chl a), chlorophyll b (chl b) and carotenoids, were measured spectrophotometrically as described by Metzner *et al*. (1965). One gram of fresh leaves was homogenized in 85 % (v/v) aqueous acetone. The extinction was measured against a blank of pure 85 % aqueous acetone at three wavelengths (663, 644 and 452.5 nm). The concentrations of the pigments (chl a, chl b and carotenoids) were calculated as µg ml^{-1} according to the following equations:

$$\text{Chl a} = 10.3\ E_{663} - 0.918\ E_{644}$$

$$\text{Chl b} = 19.7\ E_{644} - 3.87\ E_{663}$$

$$\text{Carotenoids} = 4.2\ E_{452.5} - (0.0264 \text{ chl a} + 0.4260 \text{ chl b})$$

The pigment contents were calculated as µg g^{-1} FW of leaves.

2.5. Antioxidant Enzyme Activities

The APX (EC 1.11.1.11) activity level was determined according to Asada (1992) by measuring the decrease in optical density at 290 nm as a result of the oxidation of acyl-CoA synthase using a Spectronic 601 UV spectro-photometer. The reaction mixture (3 mL) contained 50 mM ascorbic acid and 0.1 mM Ethylenediamine tetraacetic acid (EDTA) and 0.1 mL enzyme extract. The reaction was started by adding H_2O_2 to a final 1.5-mM concentration. The non-enzyme extract mixture was used as the blank. The assay was carried out according to Prochazkova *et al*. (2001). The enzyme activity was expressed as unit h^{-1} g^{-1} FW.

The CAT (EC 1.11.1.6) activity was determined by measuring the initial rate of disappearance of H_2O_2 (Aebi, 1983). The reaction mixture (3 mL) contained 10 mM of potassium phosphate buffer (pH 7) and 0.1 mL of enzyme extract. The reaction was started by adding 0.035 mL of 3 % H_2O_2. The decline in optical density at 240 nm was monitored. The non-enzyme reaction mixture was used as the blank. The CAT activity was expressed as unit h^{-1} g^{-1} FW.

The GST (EC 2.5.1.13) activity was measured according to Vontas *et al*. (2000) by observing the conjugation of 1-chloro, 2,4-dinitrobenzene (CDNB) with reduced GSH. This is indicated by an increase in the absorbance at 340 nm. One unit of enzyme conjugates 10 nmol of CDNB with reduced GSH per minute at 25°C. The reaction mixture contained 980 µl PBS (pH 6.5), 10 µL of 100 mM CDNB and 10 µL of 100 mM GSH. The GST activity was calculated using the extinction coefficient of CDNB: 0.0096 $\mu M^{-1}cm^{-1}$.

2.6. Protein Extraction and Quantification

Leaf tissue (0.5 g from each treatment) was ground on ice using a mortar and pestle with 5 mL of 10 mM potassium phosphate buffer (pH 7.0) containing 4 % (w/v) polyvinylpyrrolidone. The crude extract was centrifuged at 12,000 ×g for thirty minutes at 4°C, and the supernatant was used. The amount of protein in the extract was determined using Bradford's method (Bradford, 1976).

2.7. Protein Electrophoresis

One-dimensional SDS-PAGE was carried out according to the method described by Studier (1973) in a linear polyacrylamide resolving gel (12 %) with a stacking gel (4 %). The samples were loaded into the wells and electrophoresed at 100 V until the dye front reached the bottom of the gel. The gel was removed from the plates and shaken in staining solution (400 mL methanol, 100 mL glacial acetic acid, 500 mL distilled water and 1 g Coomassie Brilliant Blue R-250) for two hours, and was then transferred to a destaining solution (400 mL methanol, 100 mL glacial acetic acid and 500 mL distilled water) until protein bands appeared.

2.8. Amino Acid Analysis

The samples were dried, defatted and weighed to 100 mg in screw-capped tubes. Then, 5 mL of 6.0 N HCl was added. The hydrolysis tubes were attached to a system, which allowed the connection of nitrogen and vacuum lines without disturbing the samples. The tubes were placed in an oven at 110°C for twenty-four hours. The tubes were then opened, and the content of each tube was filtered and evaporated for dryness in a rotary evaporator. A suitable volume of sodium citrate buffer (pH 2.2) was added to each dried film of the hydrolyzed samples. After dissolving all of the soluble materials completely, the samples were then filtered using a 0.2-µm membrane filter, and were then ready for analysis (Baxter, 1996). The system used for the analysis was a high-performance Amino Acid Analyzer, Biochrom 20 (Auto Sampler Version) from Pharmacia Biotech, constructed at The National Center for Radiation Research and Technology (NCRRT). The chromatogram analysis was performed using an Ezchem^{TM} Chromatography Data system's tutorial and user's guide, version 6.7.

2.9. Reduced Glutathione Content

The reduced glutathione (GSH) content was extracted and determined by the method of Tanaka *et al*. (1985). Data are expressed as µg g^{-1} FW.

2.10. Macro and Micro Minerals

The macro minerals Mg^{2+} and Ca^{2+}, and the micro mineral Zn^{2+} were extracted from dried roots and shoots (including stems and leaves) according to Chapman and Pratt (1978), and were measured using an atomic absorption spectrometer in terms of mg kg^{-1} DW of the sample for Zn^{2+} and g kg^{-1} DW for Mg^{2+} and Ca^{2+}.

2.11. Pb Accumulation

The plant samples were digested with HNO_3 (65 % Merck supra pure) and $HCIO_4$ (65 % Merck supra pure) in 5:1 ratio until a transparent solution was obtained (Allen *et al*., 1986; Markert, 1996). The Pb concentration was determined using a Perkin Elmer 4300 DV Inductive Coupled Plasma and expressed as mg kg^{-1} DW.

2.12. Statistical Analyses

The experimental design was a complete random block. According to Snedecor and Cochran (1990), the averages of data were statistically analyzed using a two-way analysis of variance. Significant values were determined according to the least significant difference ($P < 0.05$) using the STAT-ITCF program (Foucart, 1982).

3. Results

3.1. Growth Parameters

Lead significantly reduced all the detected growth parameters in the kidney beans (*Phaseolus vulgaris* L.) compared to the untreated control plants (Table 1). The decrease was directly proportional to the applied Pb concentration. Maximum inhibition in growth was observed in plants that received 1000 µM Pb $(NO_3)_2$ and was calculated by 43.5 %, 52 %, 60.4 %, 72.4 %, 25 % and 75.4 % below the control value in the shoot length, root length, fresh weight of shoot and root, dry weight of shoot and root, respectively. Treatment with JA caused a substantial increase in these traits under normal as well as Pb-stress conditions. Jasmonic acid completely overcame the inhibitory effect of 100 µM Pb $(NO_3)_2$ on length, the fresh and dry weights of shoot.

Table 1. Effect of Pb $(NO_3)_2$ treatments (100 and 1000 µM) in control plants or in plants treated with JA (100µM) on growth parameters of kidney bean (*Phaseolus vulgaris* L.) plants.

Treatment	Pb $(No_3)_2$ (µM)	Shoot length (cm)	Root length (cm)	Fresh weight of shoot (g)	Fresh weight of root (g)	Dry weight of shoot (g)	Dry weight of root (g)
Reference	0	11.32±0.57	15±0.60	2.8±0.30	1.107±0.123	0.2±0.035	0.134±0.016
Controls	100	9.5±0.50	12.5±0.40	1.9±0.20	0.77±0.16	0.175±0.015	0.05±0.013
	1000	6.4±0.80	7.2±0.30	1.11±0.21	0.305±0.004	0.15±0.012	0.033±0.002
Jasmonic acid (100 µM)	0	14.0±0.50	21±1.00	4.19±0.15	2.85±0.05	0.35±0.02	0.2±0.05
	100	12.5±1.2	13±0.50	3.77±0.08	0.97±0.05	0.3±0.04	0.1±0.002
	1000	9.75±0.35	10±0.40	2.267±0.363	0.617±0.096	0.2±0.033	0.075±0.004
LSD at 0.05		2.19	1.789	0.729	0.292	0.097	N.S.

N.S. = Not significant; Data expressed as mean of ten samples ± SD.

3.2. Relative Water Content (RWC)

Changes in RWC in response to Pb toxicity in the presence or absence of JA treatment were shown in Figure 1. Pb toxicity significantly reduced RWC of kidney bean leaves. The reduction was evaluated by 20 % and 25 % in plants treated with 100 and 1000 µM of Pb $(NO_3)_2$, respectively. Leaf RWC increased in plants treated with JA and subjected to 0, 100 and 1000 µM of Pb $(NO_3)_2$ by 18.9 %, 23.1 % and 7.5 %, respectively compared to the untreated plants that received the same amounts of Pb $(NO_3)_2$. The percentage of RWC in plants sprayed with JA and subjected to 100 µM of Pb $(NO_3)_2$ was much higher than that of the untreated plants grown under normal growth conditions.

3.3. Photosynthetic Pigments

Different levels of lead stress significantly reduced both chlorophyll a and b contents, with chlorophyll b being the most affected as indicated by the increased chl a/b ratio compared to the control value. In contrast, carotenoids significantly increased by 92.3 % and 154.1 % over the control value in plants treated with 100 and 1000 µM of Pb $(NO_3)_2$, respectively (Table 2).

Jasmonic acid (100 µM) significantly increased chlorophyll a, b, carotenoids and the total pigment content compared to the untreated control plants. Their values in JA-treated plants were calculated by 2.68, 1.92, 4.24 and 2.7-fold of the control plants grown under normal conditions, respectively. In addition, JA completely alleviated the inhibitory effect of 100 µM of Pb stress on chlorophyll a and b. Interestingly, the total photosynthetic pigment content was significantly higher in the plants treated with different levels of Pb $(NO_3)_2$ alone or in combination with JA compared to the untreated control plants.

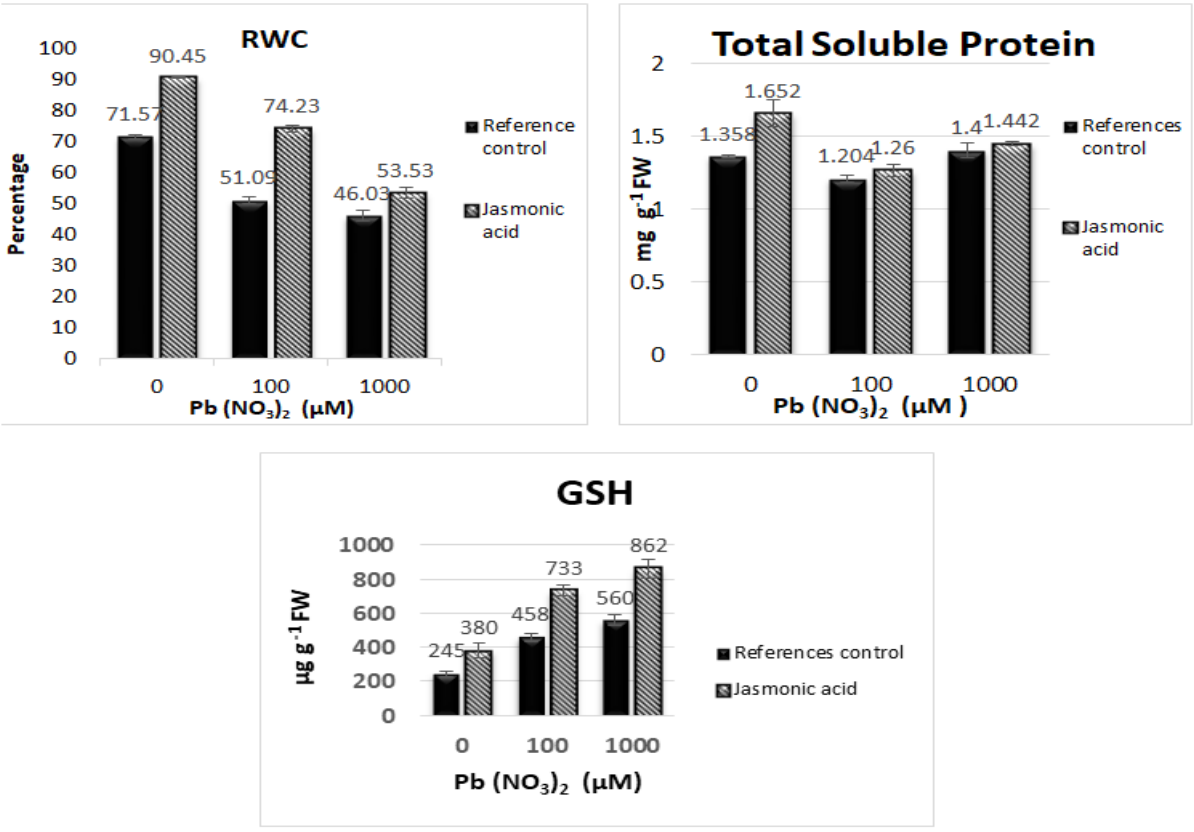

Figure 1. Effect of Pb $(NO_3)_2$ treatment (100 and 1000 µM) on control plants or plants treated with JA in terms of relative water content (RWC), total soluble protein, and glutathione content of *Phaseolus vulgaris* L. plants. Data presented as mean ± SD.

Table 2. Effect of Pb $(NO_3)_2$ treatments (100 and 1000 µM) in control plants or in plants treated with JA (100µM) on photosynthetic pigments content (µg g^{-1} FW) of kidney bean (*Phaseolus vulgaris* L.) leaves.

Treatment	Pb $(NO_3)_2$(µM)	Chlorophyll a	Chlorophyll b	Carotenoids	Chl a/Chl b	Total pigments
Reference	0	334.594±9.144	203.32±2.38	107.015±3.25	1.646±0.064	644.929±10.014
Controls	100	316.957±0.703	125.752±1.688	205.824±1.934	2.520±0.028	648.533±0.457
	1000	283.003±6.797	126.897±2.197	272.162±0.508	2.230±0.092	682.061±4.093
Jasmonic acid (100 µM)	0	897.164±8.136	389.694±0.994	453.891±3.768	2.302±0.027	1740.748±3.375
	100	500.439±1.342	205.847±1.247	312.695±2.975	2.431±0.021	1018.981±3.070
	1000	304.22±2.01	127.635±0.665	255.253±0.912	2.384±0.028	687.107±2.258
LSD at 0.05		17.895	5.082	7.800	0.169	15.052

Data expressed as mean of five samples ± SD.

3.4. Antioxidant Enzymes Activity

Data presented in Table 3 show that Pb-stress significantly increased the activity of catalase, ascorbate peroxidase, and glutathione transferase enzymes compared to the unstressed control plants. The increase in antioxidant enzymes activity was directly proportional to the applied concentration of Pb $(NO_3)_2$, and was calculated in the plants that received 1000 µM of Pb $(NO_3)_2$ by 1.9, 1.7 and 6.4-fold of the untreated control values in catalase, ascorbate peroxidase, and glutathione transferase, respectively.

Table 3. Effect of Pb$(NO_3)_2$ treatments (100 and 1000 µM) in control plants or in plants treated with JA (100µM) on antioxidant enzymes (catalase and ascorbate peroxidase) and glutathione transferase activities of kidney bean (*Phaseolus vulgaris* L.) plants.

Treatment	Pb $(No_3)_2$ (µM)	Catalase (CAT) Unit $h^{-1}g^{-1}$ FW	Ascorbate peroxidase (APX) Unit $h^{-1}g^{-1}$ FW	Glutathione transferase (GST) mM g^{-1}FW
Reference	0	2.74±0.14	9.8±0.022	451.03±6.44
Controls	100	5.02±0.15	9.83±0.122	1114.33±20.59
	1000	5.2±0.0	16.82±0.375	2895.83±00
Jasmonic	0	3.65±0.26	1.54±0.01	1650.62±0.29
acid (100	100	6.5±0.12	17.07±0.253	2587.77±00
µM)	1000	6.82±0.06	35.46±0.171	3968.75±33.33
LSD at 0.05		0.00	0.624	18.85

Data expressed as mean of three samples ± SD.

Spraying the kidney bean plants with jasmonic acid increased the level of catalase and glutathione transferase activities in the control plants as well as in the plants treated with 100 and 1000 µM of Pb $(NO_3)_2$. On the other hand, JA treatment significantly decreased the APX activity in control plants (Table 3), whereas its activity levels increased in JA-treated plants grown under 100 and 1000 µM of Pb $(NO_3)_2$ stress compared to the plants treated with Pb alone.

3.5. Protein Analysis

The total soluble protein increased in kidney bean plants sprayed with JA under normal and Pb-stress conditions compared to untreated plants grown under the same conditions (Figure 1).

Total protein was extracted from the control group and each treatment. Equal amount of protein was loaded in each lane of the SDS-polyacrylamide gel. The effect of lead nitrate (100 and 1000 µM) on the protein profiles of kidney beans in the absence or presence of jasmonic acid are shown in Figure 2. The soluble protein profiles of the control and all treatments included six common major bands and several minor bands. The main polypeptide bands are located between 10 and 200 KDa.

The electrophoretic analysis of protein patterns of the control and both concentrations of Pb (100 and 1000 µM) showed that the polypeptides with molecular weights ranging from 10 to 200 KDa were all obvious in the control (Figure 2). Lead stress increased the accumulation of certain protein bands, and completely inhibited or caused the *de novo* synthesis of others, compared with the control plants grown under normal conditions. In this respect, the 20 and 70 KDa bands were more intense accumulating in plants grown on 100 µM Pb, compared with the control. On the other hand, three protein bands with molecular weights 150, 40 and 25 KDa were completely inhibited in response to the 100 µM of Pb. This number increased to 6 (Molecular weight: 150, 120, 110, 100, 40 and 25 KDa) in the plants that received 1000 µM of Pb. A new protein band with the molecular weight 50 KDa was detected in the Pb-stressed plants, but was not found in the control plants (Figure 2)

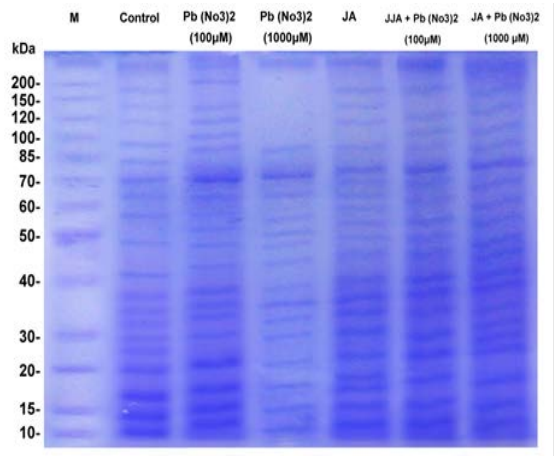

Figure 2. SDS-PAGE protein profiles of *Phaseolus vulgaris* L. treated with different concentrations and combinations of Pb $(NO_3)_2$ and JA.

Jasmonic acid caused a dual effect on the protein profile of kidney bean plants. It restored the stress inhibited proteins in the 100 to 200 KDa region, and induced the synthesis of two new protein bands in the range of (40-70 KDa).

3.6. Amino Acids Profile

Data presented in Table 4 show that the total amino acid content significantly decreased in response to Pb stress (100 and 1000 µM). On the other hand, treatment with JA significantly increased the total amino acids in plants under normal or stress conditions compared to the corresponding control. Essential amino acids including threonine, valine, methionine, isoleucine, leucine, phenylalanine and lysine significantly decreased in the plants treated with Pb $(NO_3)_2$ (100 and 1000 µM), compared to the control plants. The amounts of essential amino acids increased in response to 100 µM JA treatment whether the plants were grown under normal conditions or subjected to Pb-stress (except valine and isoleucine where JA treatment decreased their amounts under normal growth condition). Most of the non- essential amino acids followed the same pattern in response to Pb stress, with few exceptions. For example, the levels of Asp, Pro, and Arg were significantly increased in the plants treated with different concentrations of Pb and Glu level increased dramatically in the 100 µM Pb $(NO_3)_2$ -treated plants compared to the control plants. The amounts of Pro were 2 and 3.25-fold of the control value in the plants treated with 100 and 1000 µM of Pb $(NO_3)_2$, respectively. JA treatment significantly increased all the detected non-essential amino acids in the plants grown under normal conditions or subjected to Pb stress compared to the corresponding control value (except Tyr and Arg which decreased in response to JA below the corresponding control values) (Table 4). The amounts of Glu, Pro, Gly, and Cys in JA treated plants were 1.45, 2.25, 1.5 and 7-fold of those in the case of the untreated control plants.

Table 4. Effect of Pb $(NO_3)_2$ treatments (100 and 1000 µM) in control plants or in plants treated with JA (100µM) on amino acids (mg g^{-1} DW) profile of kidney bean (*Phaseolus vulgaris* L.) leaves.

Amino acids \ Treatment	Control	100 µM Pb $(NO_3)_2$	1000 µM Pb $(NO_3)_2$	Jasmonic acid (100 µM)	Jasmonic acid (100 µM)+ 100 µM Pb $(NO_3)_2$	Jasmonic acid (100 µM)+ 1000 µM Pb $(NO_3)_2$	LSD at 0.05
Essential amino acids							
Threonine (Thr)	0.65±0.05	0.5±0.02	0.35±0.08	0.7±0.02	0.6±0.03	0.5±0.05	0.218
Valine (Val)	0.7±0.2	0.35±0.01	0.35±0.02	0.6±0.02	0.5±0.01	0.7±0.031	0.097
Methionine(Met)	0.05±0.00	0.05±0.00	0.05±0.00	0.1±0.00	0.05±0.00	0.3±0.05	0.097
Isoleucine (Ile)	0.65±0.02	0.4±0.01	0.35±0.00	0.5±0.02	0.5±0.01	1.0±0.03	0.022
Leucine (Leu)	2.85±0.05	1.65±0.1	1.75±0.2	2.7±0.31	2.2±0.1	1.1±0.05	0.169
Phenylalanine(Phe)	0.7±0.01	0.55±0.05	0.55±0.01	0.8±0.04	0.8±0.01	0.9±0.05	0.138
Lysine (Lys)	0.9±0.05	0.65±0.05	0.55±0.04	1.1±0.1	1.0±0.05	0.8±0.1	0.138
Non-essential amino acids							
Aspartic acid (Asp)	0.75±0.01	1.5±0.03	1.6±0.05	1.2±0.15	1.8±0.04	1.9±0.07	0.218
Serine (Ser)	0.65±0.02	0.5±0.00	0.35±0.01	0.8±0.03	0.6±0.02	0.6±0.04	0.097
Glutamic acid (Glu)	0.55±0.02	1.5±0.05	0.35±0.00	0.8±0.00	1.7±0.1	0.6±0.03	0.097
Proline (Pro)	0.4±0.1	0.8±0.08	1.3±0.15	0.9±0.01	2.0±0.2	2.7±0.1	0.377
Glycine (Gly)	0.6±0.2	0.5±0.01	0.25±0.02	0.9±0.12	0.65±0.01	0.4±0.02	0.258
Alanine (Ala)	0.5±0.01	0.25±0.01	0.25±0.01	0.6±0.02	0.4±0.01	0.3±0.02	0.097
Cysteine (Cys)	0.15±0.03	0.11±0.00	0.05±0.01	1.05±0.05	0.5±0.04	0.4±0.02	0.258
Tyrosine (Tyr)	0.7±0.05	0.25±0.02	0.25±0.01	0.4±0.1	0.2±0.05	0.6±0.07	0.169
Histidine (His)	0.9±0.02	0.65±0.05	0.6±0.02	0.9±0.04	0.9±0.05	1.0±0.07	0.169
Arginine (Arg)	0.9±0.03	1.1±0.05	1.5±0.04	0.8±0.03	0.5±0.01	0.4±0.1	0.195
Total	12.6	11.31	10.45	14.85	14.9	14.2	0.12

Data expressed as mean of three samples ± SD.

3.7. Glutathione (GSH) Content

GSH significantly increased in response to Pb stress (Figure 1). Additional amounts of GSH were accumulated in response to JA treatment. In this respect, the GSH content was found to be higher in the case of plants treated with JA compared to the control ones (treated or untreated with Pb $(NO_3)_2$) (Figure 1).The increase in the GSH content in the JA-treated plants was estimated at 55.1 %, 60 % and 53.9 % in the plants treated with 0,100 and 1000 µM of $Pb(NO_3)_2$, respectively in comparison with the plants that are not treated with JA and grown under the same Pb-stress condition.

3.8. Mineral Ions and Pb2+ Accumulation

Kidney bean plants subjected to heavy metal stress (100 and 1000 µM of Pb $(NO_3)_2$) had significantly lower amounts of zinc, magnesium, and calcium in their shoots and roots compared to the control plants grown under normal conditions (Table 5). The levels of decrease were much more pronounced in response to the 1000 µM of Pb $(NO_3)_2$ and were evaluated as 34.6 %, 20.1 %, and 42 % below the control value in the case of shoot and 23.6 %, 5.5 %, and 42 % in the case of root in Zn, Mg, and Ca, respectively. Exogenous application of JA to heavy metal-stressed plants partially alleviated the inhibitory effect of Pb on Mg and Ca in the shoots and roots. In contrast, JA significantly decreased the Zn amounts in the control plants and those grown under different levels of Pb stress compared to the untreated plants grown under the same condition.

Plants treated with Pb $(NO_3)_2$ accumulated significantly higher amounts of Pb in their shoots and roots compared to the untreated plants. JA significantly decreased the amounts of accumulated Pb by 89 % and 95 % in the shoots, and by 48.4 % and 92.4 % in the roots of plants treated with JA + 100 and 1000 of Pb $(NO_3)_2$, respectively below those of plants treated with the same concentration of Pb $(NO_3)_2$ alone.

Table 5. Effect of Pb $(NO_3)_2$ treatments (100 and 1000 µM) in control plants or in plants treated with JA (100µM) on mineral ions content of kidney bean (*Phaseolus vulgaris* L.) plants.

Treatment	Pb $(No_3)_2$ (µM)	Zn^{2+} (mg Kg^{-1} DW)		Mg^{2+} (g Kg^{-1} DW)		Ca^{2+} (g Kg^{-1} DW)		Pb^{2+} (mg Kg^{-1} DW)	
		Shoot	Root	Shoot	Root	Shoot	Root	Shoot	Root
Reference Controls	0	58.325±0.665	46.575±1.875	9.32±0.24	5.11±0.12	25.44±0.56	15.64±0.64	0.025±0.001	2.35±0.23
	100	43.575±0.025	40.825±0.835	8.02±0.03	6.97±0.15	22.83±0.48	9.07±0.01	10.725±0.735	35.25±0.48
	1000	38.075±0.075	35.575±0.095	7.45±0.11	4.83±0.02	14.75±0.15	9.07±0.07	214.5±1.62	462.75±1.88
Jasmonic acid (100 µM)	0	51.575±1.305	40.325±0.345	11.45±0.44	7.38±0.38	28.09±0.09	16.45±0.24	0.825±0.051	1.175±0.165
	100	44.352±0.472	31.075±0.075	9.96±0.46	7.35±0.02	24.27±0.39	13.15±0.16	1.1825±0.027	18.175±0.185
	1000	37.575±0.535	30.825±0.125	8.13±0.10	7.72±0.01	19.1±0.71	11.89±0.11	10.725±0.055	35.25±0.47
LSD at 0.05		2.051	2.627	0.877	0.534	1.395	0.898	2.239	2.547

Data expressed as mean of three samples ± SD.

4. Discussion

Lead (Pb) is an example of a hazardous heavy metal that is not essential for cell metabolism, and can be easily absorbed and accumulated in different plant tissues and organs (Nas and Ali, 2018). This study found out that under Pb stress [100 and 1000 µM of Pb $(NO_3)_2$], all the detected growth parameters, chlorophyll a and b contents, were significantly ($P < 0.05$) reduced in the kidney beans (*Phaseolus vulgaris* L.) compared to the untreated control plants. The decrease was directly proportional to the applied Pb concentration. Similar results were obtained from some other studies using different concentrations of lead: root, shoot and leaf growth; fresh and dry weights are significantly decreased in *Pisum sativum* (Çimrin *et al*., 2007) and in tomato (Opeolu *et al*., 2010).

Active Pb ions can cause toxic effects and damage the photosynthetic systems in plant tissues (Yang *et al*., 2015); plant development is thus affected (Zhou *et al*. 2018). The Chlorophyll content in leaves is proved to be very sensitive to the alteration in plant oxidative status. Therefore, changes at chlorophyll level are observed in plants subjected to stress conditions (Sivaci *et al*., 2004). JA foliar spray significantly increased all the measured growth traits of kidney bean plants (including; shoot and root length, fresh and dry weights) as well as chlorophyll a and b contents compared to the untreated plants. JA completely overcame the harmful effects of up to 100 µM of Pb $(NO_3)_2$ on growth and chlorophylls. Similar results were obtained by Piotrowska *et al*. (2009) who found that chlorophyll content decreased in *Wolffia arrhiza* grown under heavy metal stress conditions compared to the control, but the exogenous application of low concentration of JA increased the chlorophyll content. Our results suggest that the application of 100 µM JA has a positive impact on photosynthetic apparatus in kidney bean plants grown under Pb stress conditions. Owing to the photosynthetic pigments' function of protection from oxidative stress and heavy metals, the increase in their content positively affects the ability of plants to adapt to environmental pollution.

On the other hand, carotenoids levels significantly ($P<0.05$) increased under Pb stress, and additional amounts accumulated when the plants were treated with JA. Carotenoids are known to serve as antioxidants due to their ability to scavenge free radicals, and to reduce cell membrane damage caused by heavy metal poisoning (Czerpak *et al*., 2006). The increased level of carotenoids induced by Pb stress treatment may be an adaptive response in the kidney bean plants under stress conditions.

Under Pb stress, RWC was markedly reduced in the kidney bean leaves compared to the control. Kastori *et al*. (1992) confirmed that excess lead damages the plant root system, and reduces water uptake resulting in an inadequate water supply to the plant shoot system (Kastori *et al*., 1992). The exogenous application of JA somehow reverses the harmful effects of Pb on RWC, JA at 100 µM increased the RWC of plants treated with 100 µM of Pb compared to the untreated control. These results show that jasmonic acid might act as a protector of the dehydration process under both control and metal stress conditions via increasing the RWC, as an attempt to protect plants against adverse conditions.

Excess Pb in cells damages plants either directly or indirectly by increasing the oxidative load caused by ROS formation (Kumar *et al*., 2011). Generally, the alleviation of oxidative stress is attributed to the increase in enzyme activity and the scavenging of ROS formed in response to the stressful condition (Foyer and Noctor, 2005). The present results show that Pb-stress significantly ($P<0.05$) increased the activity of catalase, ascorbate peroxidase, and glutathione-*S*-transferase as well as the glutathione content in the kidney bean plants. The results proved that the plants treated with Pb and JA have significantly increased the ASPX, GST activities and glutathione content [ascorbate - glutathione (Asc–GSH) cycle components] compared to the plants treated with Pb alone. The ASC–GSH cycle serves in the removal of H_2O_2, which is inevitably formed as a by-product of the normal metabolism or as a consequence of environmental stress factors (Latowski *et al*., 2010). The stimulation of Asc–GSH cycle in addition to the catalase activity in response to exogenous JA treatment proved that JA help kidney bean plants in upgrading their antioxidant capacity to scavenge more free radicals.

Lead stress increased the accumulation of two protein bands (Mwt: 20 and 70 KDa), and completely inhibited six protein bands (Mwt: 150, 120, 110, 100, 40 and 25 KDa) and caused the *de novo* synthesis of a new protein band with the molecular weight of 50 KDa compared to the control plants grown under normal conditions (Figure 2). In this respect, Kumar *et al*. (2011) detected the up-regulation of fourteen proteins in *Catharanthus roseus* in response to the Pb treatment, among which are the two HSP 70s and HSP 20. HSPs functions as molecular chaperones, the proteins which are involved in the "house-keeping" inside the cell (Sørensen *et al*., 2003). The detected up-regulated and *de-novo* synthesized proteins in response to the Pb stress are expected to help in managing the cellular activities in the plants under oxidative stress. On the other hand, the detected inhibition in other proteins indicated that Pb could increase the degradation of certain proteins. This degradation might be attributed to the binding of metals to the sulphydryl groups in these proteins, leading to the disruption of structure and / or promotion of DNA damage due to ROS accumulation (Tomas *et al*., 2014). Jasmonic acid application restored the stress-inhibited proteins and induced the synthesis of two new protein bands in the range of (70-40 KDa). Several studies reported that JA induces the accumulation of a specific set of proteins, which are called jasmonate-induced proteins (JIP). These proteins are divided into five groups on the basis of their function, that is, stress and defense, photosynthesis, carbohydrates and energy production, protein metabolism, and secondary metabolites (Sharma *et al*. 2013, Farooq *et al*. 2016).

JA showed a potential to restore the accumulation of most amino acid levels that decreased in response to the Pb $(NO_3)_2$ treatment, and significantly ($P<0.05$) increased the accumulation of other amino acids including glutamic acid, glycine, cysteine and proline (Table 5). For proline in particular, the data suggest that metal-induced proline plays a vital role in metal-stress defense. Strong experimental evidence, including work with transgenic plants and algae, indicates that proline can act as an antioxidant, metal-binding and signaling molecule (Emamverdian *et al*., 2015). Histidine, cysteine, and other

amino acids are known as a potent chelators of heavy metal ions. Their accumulation should be considered as a positive response to heavy metal stress and not as a consequence of metabolic dysregulation (Sharma and Dietz, 2006). In addition, glycine, cysteine, and glutamine availability play a key role in the GSH content (Droux, 2004).

Pb stress significantly (P<0.05) reduced the amounts of Mg^{2+} and Ca^{2+} ions, and increased the level of Pb accumulated in kidney bean shoots and roots compared to the control. The detected reduction in Ca^{2+} might be ascribed to the fact that the absorption of lead by roots occurs via the apoplastic pathway or via the Ca^{2+}-permeable channels (Pourrut *et al*., 2011).

Meanwhile, at a concentration of 100 µM, JA was able to restore the decrease in Mg^{2+} and Ca^{2+} contents that took place due to the treatment with 100 µM of Pb $(NO_3)_2$ in both the plant shoot and root. Mg^{2+} plays an important role in many metabolic processes such as being a cofactor of enzyme activity with ATP, the central atom in chlorophyll, and a stabilizer for ribosomal structure (Tanoi and Kobayashi, 2015). The stimulatory effect of JA on the chlorophyll a and b contents in plants grown under Pb stress could be attributed to its effects on chlorophyll biosynthetic pathway and ameliorating the inhibitory effects of Pb stress on the Mg ion content in plant cells.

In addition, JA significantly (P<0.05) reduced the amounts of Pb accumulated in shoots and roots of plants grown under Pb stress compared to the untreated plants grown under the same stress conditions. Reduction in heavy-metal uptake in response to exogenous application of stress hormones might be caused by the reduced transpiration rate and symplastic loading of heavy metal into xylem (Lux *et al*., 2011). The protective role of 100 µM JA against Pb toxicity in the kidney bean plants could be attributed to the JA-induced inhibition of Pb bioaccumulation which allows plant cells to maintain precise homeostatic regulation of intracellular heavy-metal levels.

Conclusion

Lead strongly inhibits kidney bean growth and chlorophyll and water content. The negative effects that lead was found to have on kidney bean plants might be attributed to the impaired uptake of macronutrients (Mg^{2+} and Ca^{2+}), inhibition of essential and non-essential amino acid levels, and the accumulation of high amounts of Pb in plant shoots and roots.

JA at 100 µM was shown to effectively protect kidney bean plants from hazardous effects resulting from Pb exposure. The mechanism of JA-induced stress tolerance in kidney beans was proved to be related to the blockade of heavy metal entry to the cell, and the stimulation of the antioxidant defense mechanisms to reduce the oxidative damage induced by Pb treatment. However, further investigations are still needed to fully explore the interaction between heavy metals and jasmonic acid.

References

Aebi, H. 1984. Catalase *in vitro*. *Methods in Enzymol.,* **105**: 121–126.

Allen SE, Grimshaw HM and Rowland AP. 1986. Chemical analysis. *In*: Moore PD, Chapman SB (Eds.), **Methods in Plant Ecology**. Oxford: Blackwell Scientific Publication, London, pp 285–344.

Ali E, Hussain N, Shamsi IH, Jabeen Z, Siddiqui MH and Jiang L. 2018. Role of jasmonic acid in improving tolerance of rapeseed (*Brassica napus* L.) to Cd toxicity. *J Zhejiang Univ Sci B*, **19 (2)**: 130-146.

Arshad M, Silvestre J, Pinelli E, Kallerhoff J, Kaemmerer M, Tarigo A, Shahid M, Guiresse M, Pradere P and Dumat C. 2008. A field study of lead phytoextraction by various scented Pelargonium cultivars. *Chemosphere*, **71(11)**:2187–2192

Asada, K. 1992. Ascorbate peroxidase, a hydrogen peroxide scavenging enzyme in plants. *Physiol Plant*, 85, 235–241.

Baxter JH. 1996. **Handbook of Food Analysis.** Marcel Dekker Inc, New York

Bradford MM. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye-binding. *Anal Biochem,* **38:** 248-252.

Chany RL and Ryan JA. 1994. **Risk Based Standards for Arsenic, Lead and Cd in Urban Soils.** Declena, Frankfurt, Germany, 130 p.

Chapman HD and Pratt FP. 1978. Methods of analysis for soils, plants and water. *Univ California, Division Agric Sci Publication*, **4034**: 50-169.

Cheong J-J and Choi YD. 2003. Methyl jasmonates as a vital substance in plant. *Trends Genet.,* **19 (7)**: 409-413.

Çimrin KM, Turan M and Kapur B. 2007. Effect of elemental sulphur on heavy metals solubility and remediation by plants in calcareous soils. *Fresenius Environ Bull*, **16(9):**1113–1120.

Czerpak, R., Piotrowska, A and Szulecka, K. 2006. Jasmonic acid affects changes in the growth and some components content in algae *Chlorella vulgaris.Acta Physiol Plant,* 28**: 195.**

Droux M .2004. Sulfur assimilation and the role of sulfur in plant metabolism: a survey. *Photosynth Res.*, **79**:331–348.

Emamverdian A, Ding Y, Mokhberdoran F and Xie Y. 2015. Heavy metal stress and some mechanisms of plant defense response. Sci World J, 2015, Article ID 756120, 18 pages,.

Fariduddin Q., Yusuf M., Hayat S and Ahmad A. 2009. Effect of 28-homobrassinolide on antioxidant capacity and photosynthesis in *Brassica juncea* plants exposed to different levels of copper. *Environ Exp Botany,* 66: **418-424.**

Farooq MA, Gill RA, Islam F, Ali B, Liu H, Xu J, He S and Zhou W. 2016. Methyl jasmonate regulates antioxidant defense and suppresses arsenic uptake in *Brassica napus* L. *Front Plant Sci*, **7**: 468.

Foyer CH and Noctor G. 2005. Oxidant and antioxidant signaling in plants: a reevaluation of the concept of oxidative stress in a physiological context. *Plant Cell Environ,* **28:** 1066–1071.

Foucart T. 1982. **Analyse Factorielle, Programmatiol sur Micro-Ordinateur**. Masson, ITCF, Paris.

Grover P, Rekhadevi P, Danadevi K, Vuyyuri S, Mahboob M and Rahman M. 2010. Genotoxicity evaluation in workers occupationally exposed to lead. *Int J Hyg Environ Health,* **213 (2)**:99–106

Hall JL. 2002. Cellular mechanisms for heavy metal detoxification and tolerance. *Exp Bot.*, **53**: 1-11.

Hassanein RA, Hassanein AA, El-din A, Salama M and Hashem HA. 2009. Role of Jasmonic acid and Abscisic acid treatments in alleviating the adverse effects of drought stress and regulating trypsin inhibitor production in soybean plant. *Aust J Basic Appl Sci.*, **3 (2)**: 904-919.

Javid MG, Sorooshzadeh A, Moradi F, Sanavy SAM and Allahdadi I. 2011. The role of phytohormones in alleviating salt stress in crop plants. *Aust J Crop Sci.,* **5** 726–734.

Kastori R, Petrovic M and Petrovic N. 1992. Effect of excess lead, cadmium, copper, and zinc on water relations in sunflower. *J Plant Nutr*, **15(11):**2427–2439.

Krämer U and Clemens S. 2005. Molecular Biology of Metal Homeostasis and Detoxification. *In*: Tamäs M and Martinoia E (Eds.), **Topics in Current Genetics**. Springer Verlag, ISBN 978-3-642-06062-5, New York, pp. 216-271.

Kumar SP, Varman PA and Kumari BDR. 2011. Identification of differently expressed proteins in response to Pb stress in *Catharanthus roseus*. *Afr J Environ Sci Technol*, **5(9):** 689-699.

Latowski D, Surówka E and Strzałka K. 2010. Regulatory Role of Components of Ascorbate–Glutathione Pathway in Plant Stress Tolerance. *In*: Anjum NA *et al* (Eds.), **Ascorbate-Glutathione Pathway and Stress Tolerance in Plants**. Springer Science and Business Media B.V.

Lux A, Martinka M, Vaculik M and White PJ. 2011. Root responses to cadmium in the rhizosphere: a review. *J Exp Bot*, **62(1):** 21-37.

Maestri H, Marmiroli M, Visoli G and Marmiroli N. 2010. Metal tolerance and hyperaccumulation. Costs and trade-offs between traits and environment. *Environ Exp Bot*, **68:** 1-13

Markert B, Herpin U, Berlekamp J, Oehlmann J, Grodinska K, Mankovska B, Suchara I, Siewers U, Weckert V and Lieth H. 1996. A comparison of heavy metal deposition in selected Eastern European countries using the moss monitoring method, with special emphasis on the Black Triangle. *Sci Total Environ*, **193**: 85 –100.

Metzner H, Rau H and Senger H. 1965. Untersuchungen Zur Synchronisier barkeep ein Zelner pigment. Mango I Mutanten Von *chlorella*. *Planta*, **65:** 186.

Nas FS and Ali M. 2018. The effect of lead on plants in terms of growing and biochemical parameters: a review. *MOJ Eco Environ Sci*, **3(4):**265–268.

Opeolu BO, Adenuga PA and Ndakidemi OO. 2010. Assessment of phytotoxicity potential of lead on tomato (*Lycopersicon esculentum* L) planted on contaminated soils. *Int J Phys Sci*, **5(2):**68–73.

Piotrowska A, Bajguz A, Godlewska-yłkiewicz B, Czerpak R and Kamin'ska M. 2009. Jasmonic acid as modulator of lead toxicity in aquatic plant *Wolffia arrhiza* (Lemnaceae). *Environ Exp Bot*, **66:** 507–513.

Poonam S, Kaur H and Geetika S. 2013. Effect of jasmonic acid on photosynthetic pigments and stress markers in *Cajanus cajan* (L.) Millsp. Seedlings under Copper Stress. *Am J Plant Sci*, **4 (4)**: 817-823.

Pourrut B, Shahid M, Dumat C, Winterton P and Pinelli E. 2011. Lead Uptake, Toxicity, and Detoxification in Plants. *Rev Environ Contam Toxicol*, **213**: 113-136.

Prochzkova D, Sairam RK, Srivastava GC and Singh DV. 2001. Oxidative stress and antioxidant activity as the basis of senescence in maize leaves. Plant Sci, 2001: 765-771.

Samota MK, Bhatt L, Garg N and Geat N. 2017. Defense induced by jasmonic acid: A review. *Int J Curr Microbiol App Sci*, **6 (5):** 2467-2474.

Seth CS, Remans T, Keunen E, Jozefczak, Gielen H, Weyens N, Vangronsveld J and Cuypers A. 2012. Phytoextraction of toxic metals: a central role for glutathione. *Plant Cell Environ*, **35 (2)**: 334-346.

Sharma SS and Dietz KJ. 2006. The significance of amino acids and amino acid-derived molecules in plant responses and adaptation to heavy metal stress. *J Exp Bot*, **57(4):**711-26

Sharma P and Dubey RS. 2005. Lead Toxicity in Plants. *Braz J Plant Physiol*, **17:** 1- 19.

Sharma R, De Vleesschauwer D, Sharma MK and Ronald PC.2013. Recent advances in dissecting stress-regulatory crosstalk in rice. *Mol Plant*, **6(2):**250-60

Sofo A, Cicco N, Paraggio M et al. 2010. Regulation of the ascorbate–glutathione cycle in plants under drought stress. *In*: Anjum NA, Umar S, Chan MT (Eds.), **Ascorbate-Glutathione Pathway and Stress Tolerance in Plants.** Springer, Dordrecht, pp 137–190.

Sivaci ER, Sivaci A and Sökmen M. 2004. Biosorption of cadmium by *Myriophyllum spicatum* L. and *Myriophyllum triphyllum* orchard. *Chemosphere*, **56:** 1043–1048.

Snedecor GW and Cochran WG. 1990. **Statistical Methods**, 11th ed. Iowa State Univ. Press, Ames, Iowa, USA.

Sørensen JG, Kristensen TN and Loeschcke V. 2003. The evolutionary and ecological role of heat shock proteins. *Ecol Lett*, **6**: 1025–1037. 10.1046/j.1461-0248.2003.00528.x

Studier FW. 1973. Analysis of bacteriophage T4, early RNAs and proteins on slab gels. *J Mol Biol*, **79**: 237-248.

Tamás MJ, Sharma SK, Ibstedt S, Jacobson T and Christen P. 2014. Heavy metals and metalloids as a cause for protein misfolding and aggregation. *Biomol.,* **4:** 252–267.

Tanaka K, Suda Y, Kondo N and Sugahara K. 1985. O_3 tolerance and ascorbate dependent H_2O_2 decomposing systems in chloroplasts. *Plant Cell Physiol*, **26:** 1425-1431.

Tanoi K and Kobayashi NI. 2015. Leaf senescence by magnesium deficiency. *Plants*, **4:** 756-772.

Vontas JG, Enayati AA, Small GJ, and Hemingway J. 2000. A Simple biochemical assay for glutathione s-transferase activity and its possible field application for screening glutathione s-transferase-based insecticide resistance. *Pestic Biochem Physiol*, **68**: 184–192.

Wasternack C and Hause B. 2002. Jasmonates and octadecanoids: signals in plant stress responses and development. *Prog Nucleic acid Res Mol Biol*, **72:** 165-221.

Xang C and Oliver DJ. 1998. Glutathione metabolic genes coordinately respond to heavy metals and jasmonic acid in Arabidopsis. *Plant Cell*, **10 (9):** 1539-1550.

Yang Y, Han X, Liang Y, Ghosh A, Chen J and Tang M .2015. The combined effects of arbuscular mycorrhizal fungi (amf) and lead (pb) stress on pb accumulation, plant growth parameters, photosynthesis, and antioxidant enzymes in *Robinia pseudoacacia* L. *PLoS ONE*, **10(12):** e0145726. https://doi.org/10.1371/journal.pone.0145726

Zhou J, Zhang Z, Zhang Y, Wei Y and Jiang Z. 2018. Effects of lead stress on the growth, physiology, and cellular structure of privet seedlings. *PLOS ONE*, **13(3):** e0191139.

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Detection and Genotyping of SEN Virus among Patients with Hepatitis and Healthy Blood Donors from Baghdad, Iraq

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Abstract

SEN virus (SENV) was discovered in 1999 as a DNA virus with hepatotropic properties. This study aims at determining the prevalence of SENV infection and genotypic characteristics in hepatitis patients and healthy blood donors. Serum samples were collected from fifty patients with a history of hepatitis B or C. In addition, fifty sera were collected from healthy blood donors as a control group. The serum samples were tested by nested polymerase chain reaction (PCR) for the detection of SENV DNA and its two genotypes (SENV-H and SENV-D). SENV was detected in 42.0 % (twenty-one out of fifty) of the hepatitis patients compared to 20 % (ten out of fifty) of the control group. SENV-H was detected in a higher prevalence than SENV-D among cases and the control group; seventeen (81 %) and nine (90 %), respectively, among cases and four (19 %) and one (10 %), respectively, among the blood donors in the control group. The prevalence of SENV in hepatitis patients was significantly higher than in healthy blood donors. There was no statistically significant relationship between SENV positivity and the mean level of liver enzyme. Hence, infection with SENV was not associated with the increased severity of the liver diseases even among HBV or HCV positive patients.

Keywords: SENV, Hepatitis B virus, Hepatitis C virus, Healthy blood donors, Prevalence, Genotype.

1. Introduction

The relatively recently discovered DNA virus SEN virus (SENV) was suspected to be significantly associated with hepatitis (Yoshida *et al.*, 2002). It was proposed that SENV belongs to a new virus family named *Anelloviridae* (Sagir*et al.*, 2004).

SENV has been described as a blood-borne pathogen that has a worldwide incidence (Sagir*et al.*, 2004). A wide range of SENV infections are reported in individuals who have a liver disease or who are human immunodeficiency (HIV)- positive, or in intravenous drug users, thalassemic patients, and patients on maintenance hemodialysis (Karimi-rastehkenari and Bouzari, 2010). SENV was previously detected in 67 % and 41 % of patients with the Hepatitis C virus (HCV) and the Hepatitis B virus (HBV), respectively. SENV was also detected in 16 % of the healthy blood donors (Kao *et al.*, 2003; El-hady *et al.*, 2006).

About 20 % of hepatic infections are not associated with hepatitis viruses (A–E) and might be attributed to other viruses. There is a very strong association between two strains of SENV (SENV-H and SENV-D) and the development of a non-A to E hepatitis infection. SENV is considered as a post-transfusion hepatitis virus. However, because the majority of SENV-infected patients do not develop hepatitis, causality is difficult to establish (Hosseini and Bouzari, 2016). Although the pathogenicity of SENV is not fully clear, SENV can undoubtedly infect patients who are already infected with other viruses. The most important high-risk persons are those infected with HBV and HCV (Kao *et al.*, 2003; Dehkordi and Doosti, 2011). A previous study indicated that SENV has a positive impact on liver pathology by decreasing liver damage, which in turn can result in a reduction of liver enzyme levels (Hosseini and Bouzari, 2016).

This study aims at determining the frequency of SENV viremia and the genotypes (SENV-D and SENV-H) by performing nested-PCR in patients with HBV, HCV or healthy blood donors, and to estimate the level of liver enzymes and risk factors.

2. Materials and Methods

2.1. Subjects

This study has a case control design. A total of one-hundred blood samples were collected from fifty patients with HBV or HCV from the Gastroenterology and Hepatology Teaching Hospital over the period from November, 2017 to March, 2018. Another fifty blood samples were collected from healthy blood donors at the Blood Donation Center in Al Imamein Al Kadhimein Medical City. The clinical characteristics of both patients and healthy individuals in the control such as (Alanine Aminotransferase (ALT), Aspartate Aminotransferase (AST), HCV-Ab, HBsAg and HBcAb) were obtained from medical records. The study has been approved by the Ethical Committee of Al-Nahrain Collage of Medicine.

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2.2. Specimens' Collection:

The serum samples were collected from all patients and individuals in the control group by venipuncture of the median cubital vein. Five mL blood samples were collected in sterile gel tubes without any anticoagulant. The samples were allowed to clot at the room temperature within one hour of collection, before centrifugation at 3,000 rpm for ten minutes, and were then stored frozen at (-44) ˚C until testing.

2.3. DNA Extraction:

The serum samples were removed from the deep freezer (-44) and were allowed to thaw at the room temperature. Viral Nucleic Acid Extraction Kit II (Cat. #VR00, Geneaid, Taiwan) was used for the isolation and purification of DNA from the samples. The procedure was done according to the manufacturer's instructions.

2.4. SEN virus DNA Amplification:

DNA amplification reactions were carried out by the nested conventional PCR according to (Hosseini and Bouzari, 2016) with modifications to optimize the results using first round primers SENV-AI-1F (TWCYCM-AACGACCAGCTAGACCT) and SENV-AI-1R (GTTTGTGGTGAGCAGAACGGA) (Alpha DNA, USA) for the SENV detection, while second round primers were SENV-D-1148F (TTTGGCTGCACCTTCTGGTT) and SENV-D-1341R (AGAAATGATGGGTGAGTGTTAGG-G) (Alpha DNA, USA) for the SENV-D genotype detection and SENV-H-1020F (CTAAGCAGCCCTAAC-ACTCATCCAG) and SENV-H-1138R (GCAGTTGA-CCGCAAAGTTACAAGAG) (Alpha DNA, USA) for the SENV-H genotype detection. For the first round reaction, the following components were mixed together in AccuPower® ProFi Taq PCR PreMix tube (Bioneer Korea): 2 µL of 10 uM/µL (SENV-AI-1F) primer and 2 µL of 10 uM/µL (SENV-AI-1R) primer, 3 µL of DNA and 13 µL of DNase free sterile water (Promega, USA), in a final reaction volume of 20 uL without calculating the volume of the lyophilized pellet of the AccuPower® PCR PreMix tubes. For the second-round reaction, the following components were mixed together in AccuPower® ProFi Taq PCR PreMix tube (Bioneer Korea): 1 µL of 10 uM/µL (SENV-D-1148F) primer and 1 µL of 10 uM/µL (SENV-D-1341R) primer or 1 µL of 10 uM/µL (SENV-H-1020F) primer and 1 µL of 10 uM/µL (SENV-H-1138R) primer, 1 µL of amplified DNA from the first PCR run, 17 µL of DNase free sterile water (Promega, USA), in a final reaction volume of 20 µL (without taking into account the volume of the lyophilized pellet of the AccuPower® PCR PreMix tubes). Cycling conditions for both the first- and second-round reactions were as follows: initial denaturation 95˚C for five minutes (1 cycle), DNA amplification by sequential denaturation of DNA at 95˚C for thirty sceonds, annealing at 60˚C for forty-five seconds, and extension at 72˚C for forty-five seconds (thirty-five cycles), and a final extension at 72˚C for five minutes (one cycle) as shown in Table 1. For visualization, the PCR amplification products were subjected to electrophoresis on 1 % agarose (Bio Basic, Canada) in 1X TBE solution (Promega, USA). The SENV-DNA positive samples showed a 349 bp band for all SENV genotypes after the first PCR round, and a 124 bp band for SENV-H or 198 bp band for SENV-D after the second PCR round (Hosseini and Bouzari, 2016). Positive control for SENV DNA was chosen after a random screening of the serum samples and confirmation by sequencing, while the negative control consisted of a reaction tube without a template DNA. Positive and negative control groups were run with each reaction.

Table 1. PCR program for both first and second round reaction.

Step	Temperature (ºC)	Time	Cycle
1	95	5 min	1
2	95	30 s	35
	60	45 s	
	72	45 s	
3	72	5 min	1

2.5. Statistical Analysis

Analysis of data was carried out using the Statistical Package for Social Sciences (SPSS) (version 19). Categorical data were presented as count and percentage, and the differences were examined by Chi-square test (X^2-test) or Fisher's exact test. On the other hand, numerical data were presented as mean ± standard deviation (SD), and evaluated by the independent sample T-test. Statistical significance was considered at a *P* value equal or less than 0.05.

3. Results

This study includes fifty hepatitis patients with HBV or HCV infection, with a mean age of 36.20 ± 13.4 years, in addition to fifty healthy blood donors as control with a mean age of 35.22 ± 9.8 years. Thirteen out of fifty (26 %) patients were hepatitis C-positive, while thirty-seven out of fifty (74 %) patients were hepatitis B- positive. Regarding gender distribution, there were twenty-four (48 %) males and twenty-six (52 %) females among the patients' group, compared to forty-seven (94%) males and three (6%) females among the control group. The mean values of the liver function test parameters i.e., ALT and AST, were higher among hepatitis patients than in healthy blood donors [50.9 ± 49.7 vs. 13.46 ± 3.840 (U/l)] and [52.6 ± 56.5 vs. 24.58 ± 7.271 (U/l)], respectively, as shown in Table 2.

Table 2. Distribution of patients and control according to age, gender and type of hepatitis infection.

Variables		Category: Patients group (n= 50)	Category: Controls group (n= 50)	Statistic
Age (mean ± SD) year		36.20 ± 13.4	35.22 ± 9.8	*P*= 0.679*
Gender	Male (n= 71)	24 (48.0 %)	47 (94.0 %)	*P*= 0.000**
	Female (n= 29)	26 (52.0 %)	3 (6.0 %)	
ALT (mean ± SD) (U/l) ALT (U/l)		50.9 ±49.7	13 .46 ±3.840	*P* =0.000*
AST (mean ± SD) (U/l)		52.6 ±56.5	24.58 ±7.271	*P* =0.001*
Type of Hepatitis	HBV	37 (74.0 %)	None	----------
	HCV	13 (26.0 %)	None	

* Using T-test at 0.05 level.
** Using Chi-square test at 0.05 level.
*** Normal values: AST 15-37 U/L, ALT 12-78 U/L

3.1. Detection of SEN Virus

SENV DNA was detected in twenty-one out of fifty (42.0 %) patients by the nested conventional PCR, while only ten out of fifty (20 %) in the control group were found to be SENV-DNA positive. The patients' group was 2.897 times more likely to be SENV-DNA positive as the control group [Odds Ratio (OR) = 2.897] as shown in Table 3 and Figure 1.

Table 3. Frequency of SENV DNA among cases and controls.

SENV DNA status	Category: Patients group (n = 50)	Category: Controls group (n = 50)	Total (%)	Statistic*
No. of positive (%)	21 (42.0)	10 (20.0)	31 (31)	X^2 = 5.657
No. of negative (%)	29 (58.0)	40 (80.0)	69 (69)	P = 0.017
Total (%)	50 (100.0)	50 (100.0)	100 (100)	

*(OR= 2.897; 95% CI, 1.19-7.07)

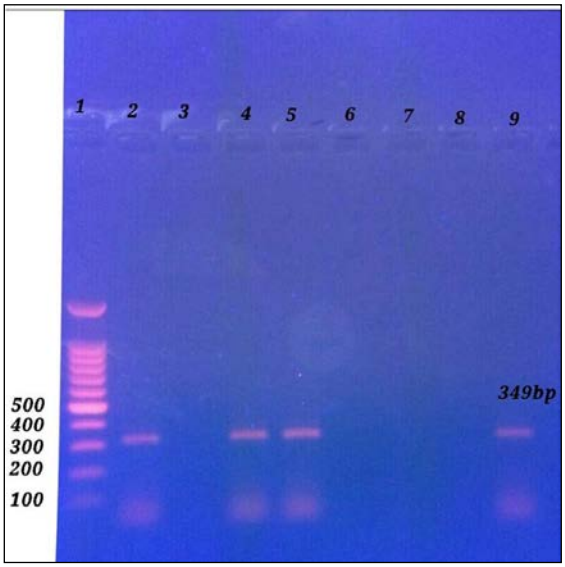

Figure 1. Gel-electrophoresis of first round PCR products on 1% agarose. Lane1, 100bp DNA marker, Lane2: positive control, Lane3: negative control, Lanes 4,5, and 9: positive samples, Lanes 6,7, and 8: negative samples.

3.2. SEN Virus Genotyping

SENV-H was detected in 81.0 % of SENV-positive patients (seventeen out of twenty-one patients; eleven HBV patients and six HCV patients) and in 90.0 % of the SENV-positive blood donors in the control. SENV-D was detected in 19.0 % of SENV- positive patients (four out of twenty-one patients; three HBV patients and one HCV patient), and in 10.0 % of the SENV-positive blood donors in the control group, as shown in Table 4 and Figures 2 and 3). In all of the studied groups, the frequency of SENV-H was higher than SENV-D. However, this difference did not reach the level of statistical significance (P>0.05).

Table 4. Detection of SENV-H and SENV-D genotype in SENV positive cases.

SENV Genotypes	Patients group (n = 50)	Controls group (n = 50)	Total (%)	Statistic
No. of SENV-H (%)	17 (81.0)	9 (90.0)	26 (83.9)	
No. of SENV-D (%)	4 (19.0)	1 (10.0)	5 (16.1)	P= 0.522
Total (%)	21 (100.0)	10 (100.0)	31(100.0)	

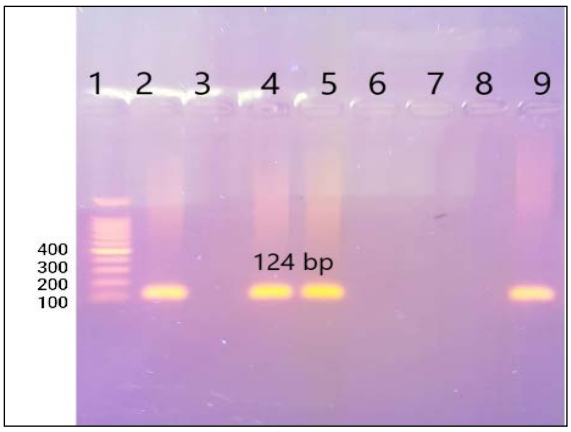

Figure 2. Gel-electrophoresis of second-round PCR products for SENV-H genotype using 1 % agarose. Lane1, 100bp DNA marker, Lane 2: positive control, Lane 3: negative control, Lanes 4, 5, and 9: positive samples, Lanes 6, 7, and 8: negative samples.

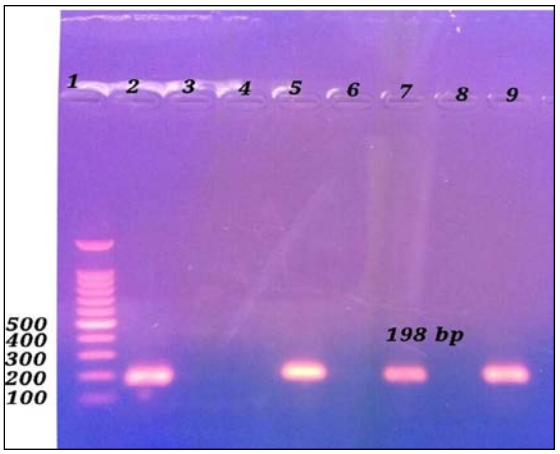

Figure 3. Gel-electrophoresis of the second-round PCR products for SENV-D genotype using 1 % agarose in TBE buffer. Lane 1, 100bp DNA marker, Lane 2: positive control for amplification, Lane 3: negative control, Lane 5, 7, 9: positive samples, Lane 4, 6, 8: negative samples.

3.3. Liver Enzymes

This study shows that the biochemical parameters i.e., liver enzymes (ALT and AST) did not significantly differ between SENV-positive and SENV-negative individuals among each of the study groups, as shown in Table 5.

Table 5. Serum ALT and AST level (U/L) in relation to SENV DNA status in patients and control groups.

Biochemical Test*	Patients group (n=50)		Statistic	Controls group (n=50)		Statistic
	SENV +Ve	SENV -Ve		SENV +Ve	SENV -Ve	
ALT (U/l)	52.48 ± 49.483	49.83 ± 50.851	t= -0.184 *P*= 0.855	14.40 ± 4.142	13.23 ± 3.779	t= -8.63 *P*= 0.931
AST (U/l)	53.62 ± 56.662	52.00 ± 57.498	t= -0.99 *P*= 0.922	29.60 ± 8.972	25.0 ± 3.80	t= -1.282 *P*= 0.230

*Values are presented as mean ± SD, Normal values: ALT (12-78) U/L and AST (15-37) U/L

3.4. Risk Factors

Concerning risk factors for individuals in this study, there was no statistically significant difference in the mean age between SENV-positive and SENV-negative individuals. Furthermore, gender, history of blood transfusion, tattooing, and surgery, did not affect the prevalence of SENV (*P*>0.05), Table 6.

Table 6. The association between SENV infection and the risk factors.

Risk factors		SENV		Total	Statistic
		Positive	Negative		
Mean of age ± SD		34.94 ± 10.692	36.06 ± 12.253	35.71 ± 11.749	t= 0.440 *P*= 0.661
Gender	Male (%)	23 (74.2)	48 (69.6)	71 (71.0)	X^2= 0.637 *P*= 0.223
	Female (%)	8 (25.8)	21 (30.4)	29 (29.0)	
	Total (%)	31 (100)	69 (100)	100 (100)	
History of Blood Transfusion	Presence (%)	2 (6.5)	2 (2.9)	4 (4.0)	X^2= 0.703 *P*= 0.402
	Absence (%)	29 (93.5)	67 (97.1)	96 (96.0)	
	Total (%)	31 (100.0)	69(100.0)	100 (100)	
History of Surgery	Presence (%)	10(32.3)	21 (30.4)	31(31.0)	X^2= 0.033 *P*= 0.855
	Absence (%)	21 (67.7)	48 (69.6)	69(69.0)	
	Total (%)	31 (100.0)	69(100.0)	100 (100)	
History of Tattooing	Presence (%)	6(19.4)	8(11.6)	14(14.0)	X^2= 1.070 *P*= 0.301
	Absence (%)	25 (80.6)	61(88.4)	86(86.0)	
	Total (%)	31(100.0)	69(100.0)	100 (100)	

4. Discussion

4.1. Detection and Genotyping of SEN Virus:

In the present study, SENV was detected in a considerable percentage of hepatitis patients with genotype H being the most prevalent. The prevalence of SENV infection was 42.0 % in the hepatitis patients and 20 % in the healthy blood donors of the control group. The SENV-prevalence rate among HCV patients is in agreement with the average prevalence rates reported in Egypt which ranged from 13.5 % to 49 % (Elsherbiny *et al.*, 2015). Globally, SENV prevalence rate was around 21 % reaching up to 69 % *(Yoshida et al., 2002; Wong et al.*, 2002). Also, results of this study are similar to those reported by Kao *et al*. (2003) who reported a high prevalence (41 %) of SENV-infection among patients with HBV. The results of Mu *et al.*(2004) showed that the prevalence rate of SENV-infection in patients with HBV was 59 % (Mu *et al.*, 2004). In this study, the percentage of SENV in the control group was 20 %. This is consistent with the range reported from other countries such as Japan (10 % to 22%) (Shibata *et al.*, 2001), Germany (8 % to 17 %) (Schröter *et al.*, 2002), Taiwan (15 %) (Kao*et al*., 2003), Italy (13 %) (Pirovano *et al.*, 2002), and Egypt (16 % to 20 %) (Mohamed *et al.*, 2011). In contrast, the results of the current study are much lower than those reported in Japan (75 %) and Isfahan (90.8 %; a central province in Iran) (Karimi-rastehkenari and Bouzari, 2010; Gerner and Wirth, 2002).

The prevalence of SENV-D/H DNA was 42.0 %. The distribution of SENV-D and SENV-H infections slightly varied between the hepatitis patients group (81.0 % for SENV-H, 19.0 % for SENV-D) and the control group (90.0 % for SENV-H, 10.0 % for SENV-D). The frequency of SENV-H was higher compared to SENV-D in both groups. However, the difference between the two groups was not statistically significant (*P*>0.05). These results were similar to those reported from Turkey and Taiwan (Serin *et al.*, 2006 and Kao *et al.*, 2002), as well as Iran (Karimi-rastehkenari and Bouzari, 2010), but are different from those reported in Egypt in which SENV-D was detected in all SENV-positive samples of the control group (Mohamed *et al.*, 2011), and from Japan which demonstrated SENV-D in 77 % and SENV-H in 15 %, of the healthy individuals (Kobayashi *et al.*, 2003).

On the whole, the SENV viremia rate differed from that reported by others (Dehkordi and Doosti, 2011; Hosseini and Bouzari, 2016; Abbasi*et al.*, 2019). The variability in SENV prevalence across different geographical regions of the world is attributed to the differences in the methods used and interactions among biological, behavioral, and social factors (El-hady *et al*., 2006). Other reasons behind this variability include differences in the quantity of SENV DNA in the sera, the use of different target sequences; un-translated region vs. open reading frame (UTR vs. ORF), differences in the sensitivities of the assay systems used or other reasons such as intravenous drug use, unsafe sexual practices, homosexuality, and professional exposure (Yoshida*et al.*, 2002).

4.2. Liver Enzymes and Risk Factors Association with SENV.

No significant relation was observed between the level of either ALT or AST or both, in the hepatitis patient or control groups, with a SENV-infection status, as shown in Table (5), suggesting that the presence of SENV did not cause an increase in the severity of liver damage in the patient group. This is similar to a pervious study from Egypt that showed a statistically insignificant difference in SENV viremia between HCV patients and HCV-related hepatocellular carcinoma patients (Kholeif and Fayez, 2008). Furthermore, Schröteret *et al.* (2002), Sagir *et al.* (2004), and Borawski *et al.* (2006) did not observe any effects on the liver enzyme levels (ALT and AST) due to the SENV infection among the hepatitis patients. In contrast, another study maintained that the levels of liver enzymes were significantly lower in the HBV patients co-infected with SENV compared to the HBV patients, indicating a positive impact of the virus on liver pathology by decreasing liver damage, and thus decreasing serum liver enzyme levels (Hosseini and Bouzari, 2016).

Most of the SEN-virus-positive individuals had a mean age of 34.94 ± 10.692 years. However, there was no significant difference in age between SENV-positive and SENV-negative among the hepatitis patients and the healthy blood donors. This is in agreement with another study that reported no significant difference between SENV-positive and SENV-negative liver patients according to age (Kholeif and Fayez, 2008). Another study reported a high prevalence of SENV among younger ages (Chiou *et al.*, 2006). No significant association with age was found in the present study.

SENV infection was found at higher proportions among males than females (74.2 % vs. 25.8 %). In spite of this, the difference was not statistically significant (P>0.05) suggesting that males and females have the same chance for SENV infection. This is in agreement with a study by Loutfy *et al*., (2009) who detected no significant differences in age and gender between SENV-positive and SENV-negative hepatitis patients. In contrast, Chiou *et al*., (2006), Kobayashi *et al.*, (2003), and Schréter *et al*., (2006) described a notable difference in the SENV prevalence according to gender with a higher proportion among the males of the SENV-positive patients**.**

The present study did not report any significant association between SENV infections and the history of blood transfusion; this may be due to the limited number of individuals having a history of blood transfusion in this study (four out of one-hundred). In addition, other studies reported that SENV was not associated with blood transfusion history (Yoshida*et al.*, 2002; Tang *et al.*, 2008) indicating that blood transfusion transmission is not the only way for people to be infected with SENV (Karimi-rastehkenari and Bouzari, 2010; Tang *et al.*, 2008). However, a previous study conducted by Mohamed *et al.* (2011) in Egypt, showed a significant difference between SENV-positive and SENV-negative patients regarding blood transfusions. The fact that SENV is also observed in healthy blood donors who had no history of blood transfusions suggests that it could be transmitted through other means than blood and injection. Possible mechanisms of transmission include fecal-oral route, saliva, amniotic fluid from SENV-positive women, breast milk, bile, and other tissues (Okamoto *et al.*, 2000).

The current study showed no significant association between SENV positivity and individuals with history of surgery. Interestingly, another study indicated that 3 % of patients who underwent an open-heart surgery were acutely infected with SENV in a complete absence of blood transfusion which suggests a nosocomial transmission of the virus as a consequence of using contaminated fomites and intravascular catheters, as well as materials associated with postoperative wounds including elastic bandages for surgical wounds. Also, instruments, equipment, and wound -dressing material may act as sources of infection as a result of contamination from blood and blood products (Umemura*et al.*, 2001).

The results of the epidemiologic studies regarding the risk of viral infections among tattooed individuals are conflicting (Deschesnes *et al.*, 2006; Jafari *et al.*, 2012). Therefore, this study also tried to investigate the relationship between SENV infection and tattooing in order to determine the risk of transmission of SENV infection. The present study didn't find an association between the history of tattooing and SENV infection in both of the study groups. These findings are consistent with a previous study from Slovaka (Schreter and Jarcuska, 2006).

In conclusion, the current study found that SENV did not seem to contribute to the pathogenesis of liver diseases among HBV- or HCV-infected patients. Further studies are still required with large samples of patients and controls, coupled with viral load quantification, to estimate the risk factors, mode of transmission and pathogenesis of SENV.

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Author contributions

All authors contributed to this manuscript by one way or another. Dr. Arwa M. Al-Shuwaikh designed, interpreted, and arranged this manuscript. Ealaf A. Khudair performed all the laboratory work and implementation of this study, and Dr. Nawal M. Farhan helped in the clinical aspects and the collection of the samples.

Conflict of Interest

There is no conflict of interest.

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References

Abbasi S, Makvandi M, Karimi G and Neisi N. 2019. The prevalence of SEN virus and occult hepatitis B (OBI) virus infection among blood donors in Ahvaz City. *Jundishapur J Microbiol*, **(7):** 3-8.

Borawski J, Kovalchuk O, Chlebińska I, Naumnik B, Rydzewska-Rosołowska A, Chyczewski L, *et al.* 2006. SEN virus infection in maintenance hemodialysis patients. *Wiad Lek*, **59(11–12):** 751-756.

Chiou SS, Huang JF, Chang TT, Hsieh MY, Dai CY, Yu ML, *et al.* 2006. SEN and hepatitis virus infections in nontransfused children and pediatric thalassemia patients with multiple transfusions in Taiwan. *Digestion*, **74(3–4):** 208-214.

Dehkordi PG and Doosti A. 2011. The prevalence of SEN virus infection in blood donors and chronic hepatitis B and C patients in Chaharmahal Va Bakhtiari province. *J. Cell Anim. Biol*, **5(8):** 182-186.

Deschesnes M, Demers S and Finès P. 2006. Prevalence and Characteristics of Body Piercing and Tattooing Among High School Students. *Can J Public Heal / Rev Can Sante'e Publique*, **97(4):** 325-329.

El-Hady SIA, Zaki SMI, El-Sayed AA, El-din MAE, Nadia M and Salamouny E-. 2006. Prevalence of SEN Virus (SEN-V) Infection

among patients on maintenance hemodialysis in Egypt. *Egyptian Journal of Medical Microbiology*, **15(1):** 99-112.

Elsherbiny NM, Hassan EA, Ahmed AO, Abd El-RehimAS, Abd *El* Rehim N and Hassany SM. 2015. Does SEN virus (SEN-V) infection affect the progression of chronic hepatitis C or B among Egyptian patients ? *Afr. J. Microbiol. Res*, **9(23):** 1504-1512.

Gerner P and Wirth S. 2002. Eigenschaften und klinische Bedeutung neuer hepatotroper Viren Hepatitis-G-Virus, TT-Virus und SEN-Virus. *Monatsschrift Kinderheilkd*, **150(1):** 19-26.

Hosseini SA and Bouzari M. 2016. Detection of SENV virus in healthy, hepatitis B- and hepatitis C-infected individuals in Yazd Province, Iran. *Iran Biomed J*, **20(3):** 168-174.

Jafari S, Buxton JA, Afshar K, Copes R and Baharlou S. 2012. Tattooing and Risk of Hepatitis B: A Systematic Review and Meta-analysis. *Can J Public Heal / Rev Can Sante'e Publique*. **103(3):** 207-212.

Kao JH, Chen W, Chen PJ, Lai MY and Chen DS. 2003. SEN virus infection in patients with chronic hepatitis C: preferential coinfection with hepatitis C genotype 2a and no effect on response to therapy with interferon plus ribavirin. *J Infect Dis*, **187(2):** 307-310.

Kao JH, Chen W, Chen PJ, Lai MYand Chen DS. 2002. Prevalence and implication of a newly identified infectious agent (SEN virus) in Taiwan. *J Infect Dis*., **185(3):** 389-392.

Karimi-rastehkenari A and Bouzari M. 2010. High frequency of SEN virus infection in thalassemic patients and healthy blood donors in Iran. *Virol J*, **7:** 1.

Kholeif L and Fayez D. 2008. SEN Virus Detection in Egyptian Patients with Chronic Hepatitis C and Hepatitis C Related Hepatocellular Carcinoma. *Egyptian Journal of Medical Microbiology*, **17(1):** 91-97.

Kobayashi N, Tanaka E, Umemura T, Matsumoto A, Iijima T, Higuchi M, Hora K and Kiyosawa K. 2003. Clinical significance of SEN virus infection in patients on maintenance haemodialysis. *Nephrol Dial Transplant*; **18(2):** 348-352.

Loutfy SA, Hafez MM, Massoud WA, Fotuh NA, Moneer MM and Zaghloul HS. 2009. SEN virus infection in Egyptian patients undergoing maintenance hemodialysis: Prevalence and clinical importance. *J Microbiol Immunol Infect*, **42(6):** 464-470.

Mohamed S, Thabit AG, Abd-El Rahman SA, Abdelmohsen EEA, Seif Eldin SS and Ghandour AMA. 2011. Prevalence of SEN virus infection in multi transfused patients in Assiut University Hospitals, Egypt. *Journal of American sciences*, **7(1):** 687-696.

Mu S-J, Du J, Zhan L-S, Wang H-P, Chen R, Wang Q-L, *et al.* 2004. Prevalence of a newly identified SEN virus in China. *World J Gastroenterol*, **10(16):** 2402-2405.

Okamoto H, Nishizawa T, Tawara A, Takahashi M, Kishimoto J, Sai T, *et al*. 2000. TT Virus mRNAs Detected in the Bone Marrow Cells from an Infected Individual. *Biochem Biophys Res Commun*, **279(2):** 700-707.

Pirovano S, Bellinzoni M, Ballerini C, Cariani E, Duse M, Albertini A, *et al.* 2002. Transmission of SEN virus from mothers to their babies. *J Med Virol*, **66(3):** 421-427.

Sagir A, Adams O, Kirschberg O, Erhardt A, Heintges T and Häussinger D. 2004. SEN virus does not affect treatment response in hepatitis C virus coinfected patients but SEN virus response depends on SEN virus DNA concentration. *World J Gastroenterol*, **10(13):** 1893-1897.

Schreter I and Jarcuska P. 2006. Detection of SEN Virus in the General Population and Different Risk Groups in Slovakia. *Folia Microbiologica*, **51 (3)**, 223-228.

Schröter M, Laufs R, Zöllner B, Knödler B, Schäfer P, Sterneck M, *et al*. 2002. Prevalence of SENV-H viraemia among healthy subjects and individuals at risk for parenterally transmitted diseases in Germany. *J Viral Hepat*, **9(6):** 455-459.

Serin MS, Tezcan S, Delialioglu N, Tiftik N, Aslan G and Emekdas G. 2006. Investigation of SEN virus genotypes D and H among blood donors in Mersin University Medical School Hospital. *Mikrobiyoloji Bülteni*, **40(1-2):** 39-45.

Shibata M, Wang RY, Yoshiba M, Shih JW, Alter HJ and Mitamura K. 2001. The presence of a newly identified infectious agent (SEN virus) in patients with liver diseases and in blood donors in Japan. *J Infect Dis*, **184(4):** 400-404.

Tang ZH, Chen XH, Yu YS and Zang GQ. 2008. Prevalence and clinical significance of SEN virus infection in patients with non A-E hepatitis and volunteer blood donors in Shanghai. *World J Gastroenterol*. **14(26):** 4204-4208.

Umemura T, Alter HJ, Tanaka E, Yeo AE, Shih JW, Orii K, *et al.* 2001. Association between SEN virus infection and hepatitis C in Japan. *J Infect Dis*, **184(10):** 1246-1251.

Wong SG, Primi D, Kojima H, Sottini A, Giulivi A, Zhang M, *et al.* 2002. Insights into SEN virus prevalence, transmission, and treatment in community-based persons and patients with liver disease referred to a liver disease unit. *Clin Infect Dis*, **35(7):** 789-795.

Yoshida H, Kato N, Shiratori Y, Shao R, Wang Y, Shiina S, *et al.* 2002. Weak association between SEN virus viremia and liver disease. *J Clin Microbiol*, **40(9):** 3140-3145.

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Population Dynamics of the Naleh Fish *Barbonymus* sp. (Pisces: Cyprinidae) in Nagan River Waters, Aceh Province, Indonesia

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Abstract

The Naleh fish *Barbonymus* sp. is among the popular commercial fresh water fishes found in Indonesia; however, the population has drastically declined over the past decade. Necessarily, a conservation program needs to be established to gather information on the population dynamics to overcome this problem. The objective of this study is to analyze the population dynamics of the Naleh fish in Nagan River. The survey was conducted from January to December, 2016. In totality, three sampling locations were selected based on information from local fishermen. The Naleh fish was sampled using gillnets (mesh size 0.5 and 1.0 inches) and casting nets (mesh size 1.5 and 2.0 inches). A total of 761 fish samples were collected for the study. The von Bertalanffy (von Bertalanffy growth function) growth parameters were utilized to analyse the population dynamics of *Barbonymus* sp., using FISAT II (FAO-ICLARM Stock Assessment Tools-II). The results show the following population dynamics: Asymptotic length (L_∞) was 160.07mm, coefficient of growth (K) = 0.73 year^{-1}, growth performance index (Ø) = 4.27 year^{-1}, time at which length equals zero (t_0) = -0.022 year^{-1}, growth and age (Lt) = 2.55 year^{-1}, and optimum length of catch (L_{opt}) = 89.9mm. In addition, the total mortality rate (Z) was 2.802 year^{-1} with a natural mortality rate (M) =0.921 year^{-1}, fishing mortality rate (F) =1.88 year^{-1}, and exploitation rate (E) =0.67 year^{-1}. The conclusion has been drawn based on the E value analysis which displays that the exploitation rate of the Naleh fish has surpassed the sustainable limit based on the value of L_∞, K, t_0, Lt, L_{opt} of the dominant one-year-old fishes caught.

Keywords: von Bertalanffy, Growth, Dynamics, *Barbonymus*, Nagan Raya waters

1. Introduction

Nagan Raya is located in the western part of Aceh Province, Indonesia. This district houses three main rivers, namely Nagan River, Lamie River, and the Seumanyam River. It also has a large abundance of freshwater resources because of the vast waters present. According to Muchlisin *et al.* (2015), there are at least seventy-three species of freshwater fishes found in these rivers. The previous study by Muchlisin and Siti-Azizah (2009) reported that a total of 114 species of freshwater and brackish water fishes were recorded from several parts of Aceh province, Indonesia. Of these, forty-six species were categorized as fish suitable for consumption, seventeen species had aquaculture potency, and ten other species had ornamental fish potency (Muchlisin, 2013). One of the promising candidates for aquaculture is the Naleh fish *Barbonymus* sp. Based on the biometric analysis showed that the morphology of the Naleh fish is close to *the B. gonionotus* (Batubara *et al*., 2018), but the genetic analysis using *Mitochondrial Cytochrome c Oxidase Subunit I (COI)* or commonly known DNA barcoding revealed the Naleh fish is not a synonymous to *B. gonionotus*, and indicates a cryptic species (Batubara, 2019); Therefore, an intensive study is being performed to validate the taxonomic status of this fish.

The *Barbonymus* has several local names, for instance, it is called Naleh and Balee and Tawes in Aceh, Sulawesi and Java, respectively (Isa *et al*., 2012). While, Malaysian people call it lampam, and it is called Thai sharpunti in Bangladesh (Mondol *et al*., 2005; Mollah *et al.*, 2011; Hossain *et al*., 2016; Reza *et al.*, 2017). Additionally, it is internationally called the silver tinfoil barb (Fish Base, 2018). This species is a major target for fishermen in the inland waters of the Nagan Raya District.

However, apart from being considered as food for consumption, the Naleh fish has a great potency as an ornamental fish, because of its attractive colours, especially on its caudal and ventral fins (Chheng *et al*., 2004; Gante *et al*., 2008; Eslamloo *et al*., 2012; Isa *et al*., 2012; Muchlisin *et al*., 2015). Its diversity in use makes it a main target for local fishermen in Nagan Raya, Indonesia leading to a decreased population due to overfishing. Besides overfishing, the Naleh population is also affected by ecological destruction of the Nagan River watershed due to deforestation, sand and gold mining in the upper stream. According to Brierley and Kingsford (2009), environmental degradation has negative impacts and may inhibit the process of fish replenishment. According to

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Maceda (2013), freshwater ecosystems face higher damage threats compared to other ecosystems in the world. Based on the International Union for the Conservation of Nature (IUCN), several species within *Barbonymus*, for example *B. gonionotus* is categorized as 'Least Concern' (Thinh *et al.*, 2012). However, the current population of the Naleh fish *Barbonymus* sp. in the Nagan Raya waters has decreased over the years (Personal communication with local fishermen of Nagan Raya). Accordingly, there is a need to evaluate the conservation status of the Naleh fish.

Information on the population dynamics is vital in determining the conservation status of fish (Lorenzen, 2005; Kinzey and Punt, 2008; Gislason *et al.*, 2010). Presently, the studies on the population dynamics of fishes focused on marine fishes; for example, Arrafi *et al.* (2016) and Jayabalan *et al.* (2014) studied the population dynamics of the Indian mackerel in western Aceh waters, Indonesia, and the Sohar Coast of Oman respectively. Al-Marzouqi *et al.* (2012) also studied the population dynamics of *Argyrops filamentosus* in Oman sea, the *Katsuwonus pelamis* in the Indian Ocean (Adam and Sibert, 2002), and *Gerres oblongus* in Laguna Jaffna, Sri Lanka (Shutharshan and Sivashanthini, 2011). Besides fishes, the study of the population dynamics has also been conducted on squids (*Sepioteuthis lessoniana*) from the South Sea of Sri Lanka (Charles and Sivashanthini, 2011) and shrimps in the Ivorian sea (Yacouba *et al.*, 2014). However, information on freshwater fish, especially from the tropical waters, was not available during the study. Hence, the objective of the present study is to analyze the population dynamics of the Naleh fish in Nagan River, Aceh Province, Indonesia.

2. Methods

2.1. Time and Location

The study was conducted in the Nagan Raya River from January to December 2016. The fish samples were analyzed in the Laboratory of Ichthyology, Faculty of Marine and Fisheries, Syiah Kuala University, Banda Aceh. The fish was taxonomically identified according to Kottelat *et al.* (1993).

2.2. Sampling Procedure

The sampling was conducted in three locations along the Nagan Raya River °16'25.25"N and 96°24'22.34"E; 4°17'4.73"N and 96°25'56.83"E; 4°16'48.49"N and 96°27'8.50"E) (Figure 1), while the sampling points were the locations suspected to contain a lot of Naleh fish based on information from local fishermen. The sampling was conducted two times weekly from 08.00 AM to 16.00 PM for twelve months. The fish was caught using casting nets (mesh size 0.5 and 1.0 inches), gillnets (mesh size 1.5 and 2.0 inches), and fishhooks. The sampled fishes were kept in an icebox (4^{o}C), and were transported to the laboratory of Syiah Kuala University for further analysis.

In the laboratory, the fish samples were measured to obtain a standard length nearest to mm using a digital caliper (Mitutoyo, CD-6CS. Error = 0.01 mm), and were weighed to measure the total body weight nearest to gram using a digital balance (Toledo, AB-204. Error= 0.01 g). Finally, the sample was preserved in a 10 % formalin solution.

2.3. Parameters Calculation

2.3.1. Length Frequency

The length frequency measurements were carried out to determine the number of fish based on the length class. For this purpose, the maximum and minimum length, mean, number of classes, intervals, differences, and class width were calculated. The length frequency analysis refers to Jin *et al.* (2015) as follows:

$Fi = ni/N_X 100$

Where Fi is length frequency (%), *ni* is the total number of fish at class length -*i*, and N is the total fish sample.

2.3.2. von Bertalanffy Growth Function

The von Bertalanffy growth function was analyzed using FISAT II (FAO-ICLARM Stock Assessment Tools-II). The growth parameter was calculated using the ELEFAN-I method. This calculation gave an asymptotic length value (L_∞) and coefficient of von Bertalanffy growth function (K). Moreover, the theoretical age at fish length-0 was calculated based on Pauly (1980) as follows: Log (t_0) = -0.3952-0.2752 Log (L_∞)-1.038-Log (K). Where t_0 is the theoretical age at fish length-0, L_∞ is asymptotic length, and K is growth co efficient.

2.3.3. Total Mortality Rate

The total mortality rate (Z) was calculated using FISAT II based on Length-coverted Catch Curve method.

2.3.3.1. Natural Mortality Rate

The natural mortality rate (M) was calculated based on Pauly (1980):

$Log(M)=-0.0066-0.279Log(L\infty)+0.6543Log(K)+0.4634Log(T)$

Where M is the natural mortality rate, L∞ is infinity or asymptotic length, K is the growth coefficient, and T is the average temperature of the waters/habitat.

2.3.3.2. Fishing Mortality Rate

The fishing mortality rate (F) was calculated based on Pauly (1980) as follows:

$F = Z-M$

Where Z is the total mortality rate, and M is the natural mortality rate.

2.3.3.3. Exploitation Rate

The exploitation rate (E) was calculated based on Sparred and Venema (1992) as follows:

$E = F/Z$

Where E is the exploitation rate, F is the fishing mortality rate, and Z is the total mortality rate.

2.3.4. The Optimum Length of the fish caught and recruitment pattern

The optimum length of the fishes caught (L_{opt}) was calculated based on Froese and Binohlan (2000) as follows: $L_{opt} = 3*L_\infty/(3+M/K)$, where L_{opt} is the optimum length of the fish caught, M is the natural mortality rate, $L_\infty$is infinity or asymptotic length, and K is the growth coefficient. The recruitment pattern was performed using FISAT II by inputting the L_∞, K and t_0 values into the growth parameter inputs and graph menu.

3. Results

A total of 761 samples of *Barbonymus* sp. were recorded throughout the study period (twelve months). The fish samples were divided into eleven length classes. The minimum length was 48mm, while the maximum length was 167.07 mm, and the average length was 116.93 mm (Figure 2a). The range was 99 mm with an interval of 49.5mm. A large proportion of the fish samples (31.67 %) had a class length of 88-97 mm and an estimated age of one year (Figure 2a, Figure 3b). The results showed that the number of fish decreased with the increasing class length (Figure 2a); therefore, the optimum class length for fishing was 88-97 mm.

The results revealed that the growth coefficient value (K) was 0.730 year^{-1}which indicates that the growth rate reaches 73 mm year^{-1}. The infinity or asymptotic length (L_∞) showed that the maximum length of the Naleh fish was 160.07 mm (Figure 3a).

The analysis of the growth length and age of the samples revealed that the age of the fish samples ranged between one and five years, while the fish attained their maximum length at about five years (Figure 3b). The mortality rate is about 2.802 year^{-1}and is comprised of the natural mortality rate (0.921 year^{-1}), fishing mortality rate (1.880 year^{-1}), and the exploitation mortality rate (0.670 year^{-1}) (Table 1). The recruitment pattern analysis shows that recruitment occurred over the year, with the higher recruitment occurring in April (15.32 %), and the lowest recruitment occurring in August (2.79 %) (Figure 4b). These data indicate that the Naleh fish spawns over the year except in December (Figure 4b). However, this finding should be confirmed with the gonadosomatic index of the fish, and this study is currently ongoing.

Table 1. Population dynamics of the Naleh fish *Barbonymus* sp. in the Nagan Raya River, Aceh Province, Indonesia

No.	Parameters	Value
1	Infinity or Asymptotic length (L_∞)	160.07 mm
2	The optimum fishing length (L_{opt})	89.9 mm
3	Growth coefficient (K)	0.730 year^{-1}
4	Total mortality rate (Z)	2.802 year^{-1}
5	Natural mortality rate (M)	0.921 year^{-1}
6	Fishing mortality rate (F)	1.880 year^{-1}
7	Exploitation rate (E)	0.67 year^{-1}

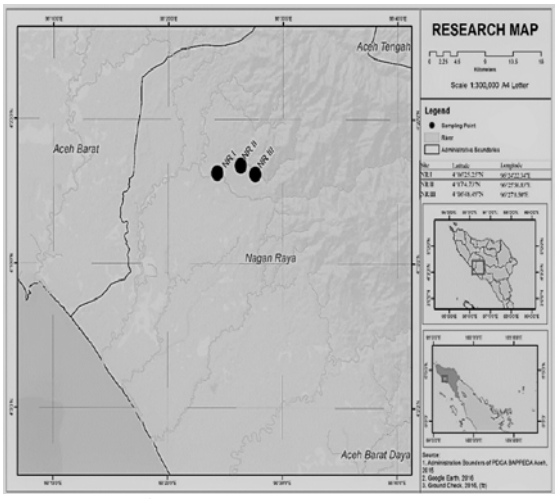

Figure 1. Map of the Nagan Raya District, Aceh Province, Indonesia showing the location sampling (black dots)

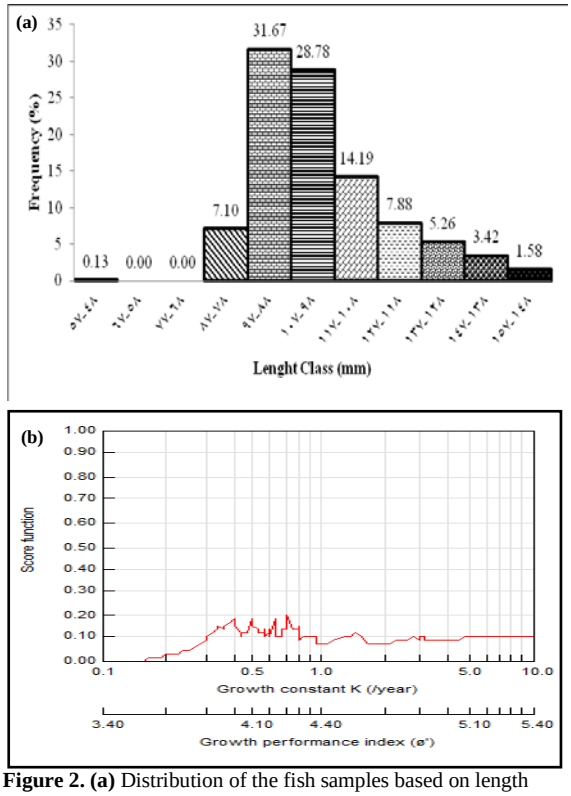

Figure 2. (a) Distribution of the fish samples based on length classes (N= 761) with the optimum length (L_{opt}) of 89.9 mm, **(b)** the estimation of the growth coefficient (K) was 0.730 year^{-1} and growth capable index (Ø) was 4.27 year^{-1} of the Naleh fish *Barbonymus* sp. in Nagan Raya River, Aceh Province, Indonesia

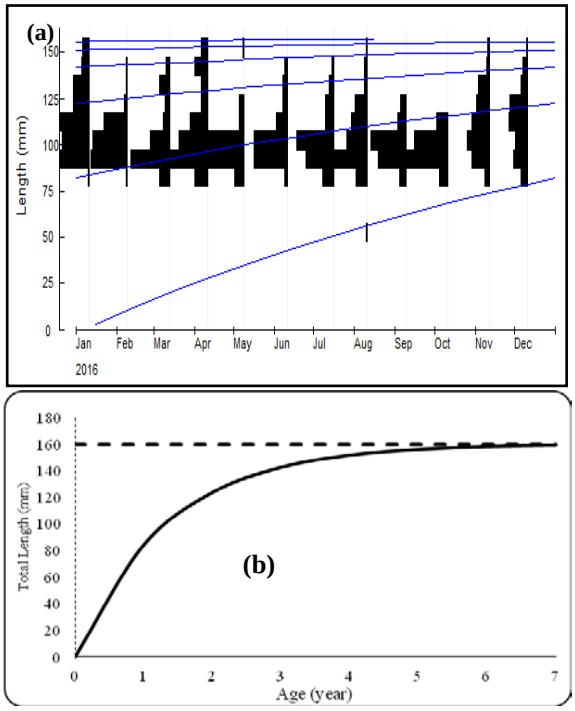

Figure 3. (a) Growth parameters estimation (L∞ = 160.7 cm and K = 0.730 year^{-1}); **(b)** the curve of the length growth and age estimation (t_0 = -0.022 and Lt = 2.55 year^{-1}) of the Naleh fish *Barbonymus* sp. in the Nagan Raya River, Aceh Province, Indonesia

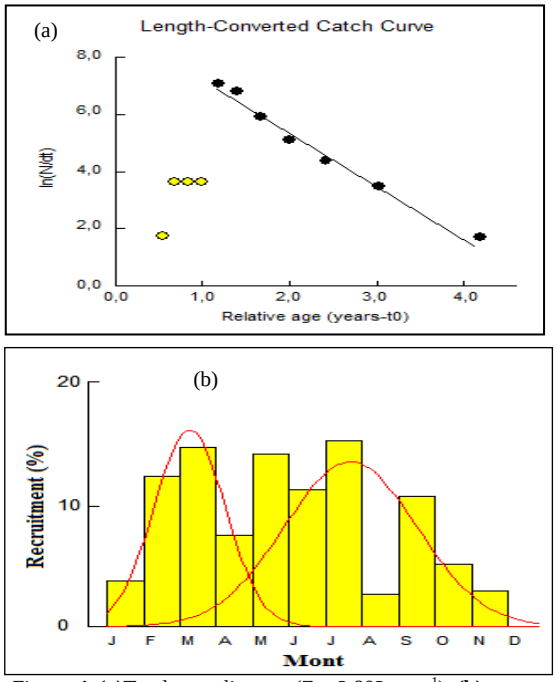

Figure 4. (a) Total mortality rate (Z = 2.802 year^{-1}); **(b)** Percentage of recruitment every month for one year of the Naleh fish *Barbonymus* sp. in the Nagan Raya River, Aceh Province, Indonesia

4. Discussion

The study shows that the majority of the fish samples were between the class length of 88-97 mm, and the optimum fish length is 89.9 mm, indicating that the length was within the limit. The study also revealed that most of the samples were one-year-old (Figure 3b). The estimation of the length to the age is about 2.55 mm year^{-1} with the infinity or asymptotic length as 160.07 mm. The results showed only few class lengths being above 88-97mm. This indicates that only few Naleh fish attained sizes above 97mm. The results are in accordance with the statement of Tyrrell *et al*. (2011) that the population of fish decreases with the increasing length because of the increase in the probability of mortality due to fishing and diseases.

In comparison, the asymptotic length of the Naleh fish *Barbonymus* sp. in the Nagan River is lower compared to that of the fish in the Mekong River, Cambodia (Chheng *et al.*, 2004) and the *B. schwanenfeldii* in Pedu Dam, Malaysia (Isa *et al*., 2012) with an infinity length of 160.07mm and 309.5mm, respectively. According to Froese and Binohlan (2000), the fish population is threatened if the fish harvest is small. This may be attributed to ecological degradation and overfishing. This indication is in agreement with several previous studies in *Barbonymus* from different locations. For instance, Hardjamulia *et al.* (1988) reported that the size of harvested *B. gonionotus* decreased from 405mm in 1999 to 340 mm in 2009 (Sastrawaha and Pilasamorn, 2009), then, it continued to decrease to 211 mm in 2010 (Garcia, 2010), and 209mm in 2016 (Hossain *et al.*, 2016) and as recorded in this study to 160.07mm in 2016.

The total mortality of the Naleh fish in the Nagan River is 2.802 year^{-1} with a fishing mortality of 1.880 year^{-1} which is higher than the natural mortality rate (0.921 year^{-1}). Moreover, the ratio M (0.921 year^{-1}) and K (0.730 year^{-1}) was 1.263; this value indicates that the Naleh fish population is threatened by intensive and unfriendly fishing practices, and the destruction of habitat as observed during the study. A similar phenomenon was also reported in Depik fish *Rasbora tawarensis* the endemic species in Lake Laut Tawar, Aceh Province, Indonesia (Muchlisin *et al*., 2011). According to Al-Marzouqi *et al.* (2012), the normal ratio M/K ranges from 1.0 to 2.5. The exploitation rate (E) of the Naleh fish in this study was 0.67 year^{-1}, which means that the total mortality was 67 %, as a result of fishing activities excluding natural mortality. The optimal value of exploitation rate (E) is 0.5 (Jayabalan *et al.*, 2014) with the estimation that the sustainable yield is optimized at F = M (Gulland, 1971). According to Isa *et al.* (2012), if the exploitation rate (E) is higher than 0.5 year^{-1},this indicates that the new recruitment has a negative correlation with the E value (overfishing).

Sparre and Venema (1992) stated that the natural mortality rate is influenced by predation, diseases, stress, spawning, and old age. The study revealed that the value of the rate of exploitation was 0.670 year^{-1}. This value was higher than the recommended maximum exploitation of 0.5 year^{-1}, which indicates that the exploitation has passed the limit. This is supported by Gabche and Hockey (1995) who explained that if the exploitation rate is higher than 0.5 year^{-1}, it means that the exploitation has crossed the sustainable exploitation limit. According to Bostford *et al.* (1997), overfishing occurs when the fishing activities supersede the recruitment capacity. Overfishing is a major problem worldwide because it can potentially lead to extinction, and a reduced biodiversity of the fishes (Coleman and Williams, 2002).

Furthermore, the recruitment pattern of the Naleh fish occurs over the year except in December, and the peak of

recruitment is in July. Therefore, more studies on reproductive biology are needed to confirm this finding. A similar trend is also reported by Jasmine and Begum (2016) that *B. gonionotus* in the Padma River, Bangladesh spawns from April to July with the peak season being in June.

5. Conclusion

It can be concluded that the recorded maximum length of the Naleh fish was 89.9 with an age estimation of one year. The optimum fish length was 160.07mm, and this length can be attained when the fish is at least five years old. The exploitation rate (E = 0.67 year^{-1}) has passed the sustainable limit (overexploitation), which means that overfishing already exists.

References

Adam MS and Sibert JR. 2002. Population dynamics and movements of skipjack tuna (*Katsuwonus pelamis*) in the Maldivian fishery: analysis of tagging data from anadvection-diffusion-reaction model. *Aquat Liv Res*, **15**: 13-23.

Al-Marzouqi A, Jayabalan N, Al-Nahdi A and Al-mamry J. 2012. Length-based stock assessment of the Soldierbream Argyrops filamentosus (Valenciennes, 1830) from the Arabian Sea off Oman. *Thalassas*, **28(1):** 57-67.

Arrafi M, Ambak AM, Rumeaida PM and Muchlisin ZA. 2016. Biology of Indian Mackerel, Rastrelliger kanagurta (Cuvier,1817) in the Western Waters of Aceh. *Iranian J Fish Sci*, **15(3):** 957-972.

Batubara AS, Muchlisin ZA, Efizon D, Elvyra R, Fadli N and Irham M. 2018. Morphometric variations of the Genus Barbonymus (Pisces, Cyprinidae) harvested from Aceh Waters, Indonesia. *Fish Aquat Life*, **26**: 231-237.

Batubara AS. 2019. *Bioecology and genetic of the Naleh fish (Barbonymus sp.) harvested from Nagan River, Nagan Raya District, Aceh Province, Indonesia*. Ph.D thesis, Syiah Kuala University, Banda Aceh.

Bostford LW, Castilla JC and Peterson CH. 1997.The management of fisheries and marine ecosystems. *Science*, **277**: 509-515.

Brierley AS and Kingsford MJ. 2009. Impacts of climate change on marine organisms and ecosystems. *Current Biol*, **19(14):** 602-614.

Charles GA and Sivashanthini K. 2011. Population dynamics of squid sepioteuthis lessoniana (Lesson, 1830) from the Northern Coast of Sri Lanka. *J Fish Aquat Sci*, **6(1):** 74-84.

Chheng P, Baran E and Touch BT. 2004. Synthesis of all published information on Java barb *B. gonionotus* ("trey chhpin) based on Fishbase 2004. Worldfish Center and Inland Fisheries research and Development Institute, Phnom Phenh, Cambodia. 20 pp.

Coleman FC and Williams SL. 2002. Overexploiting marine ecosystemengineers: potential consequences forbiodiversity. *Trends Ecol Evol*, **17(1):** 40-44.

Duffy WG. 1998.Population dynamics, production, and prey consumption of fathead minnows (Pimephales promelas) in prairie wetlands: a bioenergetics approach. *Can J Fish Aquat Sci*, **54**: 15-27.

Eslamloo K, Morshedi V, Azodi M, Ashouri G, Ali M and Iqbal F. 2012. Effect of starvation and re-feeding on growth performance, feed utilization and body composition of Tionfoil Barb (*B. schwanenfeldii*). *World J Fish Mar Sci*, **4(5):** 489-495.

Fishbase. 2018. "Genus *Barbonymus*." Available via http://www.fishbase.org/Nomenclature/ScientificNameSearchList.php. (Sept.8th, 2018).

Froese R and Binohlan C. 2000. Empirical relationships to estimate asymptotic length, length at first maturity and length at maximum yield per recruit in fishes with a simple method to evaluate length frequency data. *J Fish Biol*, **56**: 758-772.

Gabche CE and Hockey HUP. 1995. Growth, mortality and reproduction of *Sardinella maderensis* (Lowe, 1841) in the artisanal fisheries off Kribi, Cameroon. *Fish Res*, **24**: 331-344.

Garcia LMB. 2010. Species composition and length-weight relationship of fishes in the Candaba wetland on Luzon Island, Philippines. *J Appl Ichthyol*, **26**: 946-948.

Gante HF, Moreira LDC, Micael J and Alves MJ. 2008. First Record of *B.schwanenfeldii* (Bleeker) in the Iberian Peninsula. *J Fish Biol*, **72(1):** 1089-1094.

Gislason H, Daan N, Rice JC and Pope JG. 2010. Size, growth, temperature and the natural mortalityof marine fish. *Fish and Fisher*, **11**: 149-158.

Gulland JA. 1971. The fish resources of the ocean. Fishing News (Books) Ltd., West By EET, for FAO. 255 pp.

Hardjamulia A, Setiadi KE and Rabegnatar NS. 1988. Some biological aspects of the predominant fish species in the Jatiluhur Reservoir, West Java, Indonesia. Reservoir Fishery Management and Development in Asia. International Development Research Center, Ottawa, Canada. Page 104.

Hossain MY, Hossen MA, Islam MM, Pramanik MNU, Nawer F, Paul AK, Hameed HMA, Rahman MM, Kaushik G and Bardoloi S. 2016. Biometric indices and size at first sexual maturity of eight alien fish species from Bangladesh. *Egypt J Aquat Res*, **42**: 331-339.

Isa MM, Md-Shah AS, Mohd-Sah SA, Baharudin N and Abdul-Halim MA. 2012. Population dynamics of Tinfoil Barb, *B. schwanenfeldii* (Bleeker, 1853) in Pedu Reservoir, Kedah. *J Biol Agric Healthcare*, **2(5):** 55-69.

Jasmine S and Begum M. 2016. Biological aspects of *Barbonymus gonionotus* (Bleeker, 1849) in the Padma River, Bangladesh. *Int J Fish Aquat Studies*, **4(5):** 661-665.

Jayabalan N, Zaki S, Al-Kiyumi F, Al-Kharusi L and Al-Habsi S. 2014. Age, growth and stock assessment of the Indian mackerel *Rastrelliger kanagurta* (Cuvier, 1817) along the Sohar coast of Oman. *Indian J Fish*, **61(1):** 1-6.

Jin S, Yan X, Zhang H and Fan W. 2015. Weight–length relationships and Fulton's condition factors of skipjack tuna (*Katsuwonus pelamis*) in the western and central Pacific Ocean. *Peer J*, **9**: 1-11.

Kinzey D and Punt AE. 2008 Multispecies and single-species models of fish population dynamics: comparing parameter estimates. *Nat Res Model*, **22(1):** 67-104.

Kottelat M, Whitten AJ, Kartikasari SN and Wirjoatmodjo S. 1993. Freshwater fishes of Western Indonesia and Sulawesi. Periplus Editions Limited, Thailand.

Lorenzen K. 2005.Population dynamics and potential of fisheries stock enhancement: practical theory for assessmentand policy analysis. *Phil Trans Royal Soc B*, **360**: 171-189.

Maceda AV. 2013. Toward the conservation of freshwater fish: Iberian River as an example of threats and management practices. *Rev Fish Biol Fish*, **23(1):** 1-22.

Mollah MFA, Moniruzzaman M and Rahman MM. 2011. Effects of stocking densities on growth and survival of Thai Sharpunti (*Barbonymus gonionotus*) in earthen ponds. *J Bangladesh Agric Univ*, **9(2)**: 327-338.

Mondol MR, Dewan S, Hossain MA, Asaduzzaman M, Islam MA and Rozario UA. 2005. Food and feeding habits of *Puntius gonionotus* (Thai Sarpunti) in Rice Field. *Pakistan J Biol Sci*, **8(3):** 386-395.

Muchlisin ZA and Siti-Azizah MN. 2009. Diversity and distribution of freshwater fishes in Aceh Waters, Northern Sumatera, Indonesia. *Int J Zool Res*, **5(2)**: 62-79.

Muchlisin ZA, Fadli N, Rudi E, Mendo T and Siti-Azizah MN. 2011. Estimation of production trend of the depik, Rasbora tawarensis (Teleostei, Cyprinidae), in Lake Laut Tawar, Indonesia. *AACL Bioflux*, **4**: 590-597.

Muchlisin ZA. 2013. Potency of freshwater fishes in Aceh waters as a basis for aquaculture development programs. *J Iktiol Indonesia*, **13**: 91-96.

Muchlisin ZA, Akyun Q, Rizka S, Fadli N, Sugianto S, Halim A and Siti-Azizah MN. 2015. Ichthyofauna of Tripa Peat Swamp Forest, Provinsi Aceh, Indonesia. *Check List*, **11(2):** 1560.

Pauly D. 1980. On the interrelationships between natural mortality, growth parameters and mean environmental temperature in 175 fish stock. *J Cons Int Explor Mer*, **39**: 175-192.

Reza AHMM, Rakhi SF, Hossen MS and Hossain Z. 2017. Organ specific histopathology and brain Acetylcholinesterase inhibition inrohu, *Labeo rohita* and silver barb, *Barbonymus gonionotus*: effects ofthree widely used organophosphate pesticides. *Turkish J Fish Aquat Sci*, **17**: 819-830.

Satrawaha R and Pilasamorn C. 2009. Length-weight and length-length relationships of fish species from the Chi River, northeastern Thailand. *J Appl Ichthyol*, **25**: 787–788.

Shutharshan S and Sivashanthini K. 2011. Growth characteristics of slender silver-biddies *Gerres oblongus* (Pisces: Perciformes) from the Jaffna Lagoon, Sri Lanka. *J Fish Aquat Sci*, **6(6):** 592-603.

Silva KHGM. 1991. Population dynamics and production of the rocky stream-dwelling fish *Garra ceylonensis* (Cyprinidae) in Sri Lanka. *J Trop Ecol*, **7**: 289-303.

Sparre P and Venema SC. 1992. Introduction to tropical fish stock assessment. Part 1. Manual FAO Fish. Tech. Pap. 376 pp.

Thinh DV, Van NS and Nguyen THT. 2012. "*B. gonionotus*. The IUCN Red List of Threatened Species 2012. Available via " http://dx.doi.org/10.2305/IUCN.UK.2012-1.RLTS.T166914A1151554.enhttp://dx.doi.org/10.2305/IUCN.UK.2012-1.RLTS.T166914A1151554.en. (Nov. 8th, 2017).

Tyrrell MC, Link JS and Moustahfid H. 2011. The importance of including predation in fish population models:Implications for biological reference points. *Fish Res*, **108**: 1-8.

Yacouba S, Zacharie S and Joanny T. 2014. Biological aspects and yield of the shrimp *Parapenaeus longirostris*, West Africa. *Int J Agric Pol Res*, **2(4):** 132-140.

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The Effect of Rh2 Phenotype on Cytotoxic T- Cell Counts

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Abstract

Sub-Saharan Africa and the Caribbean countries bear a disproportionate burden of the HIV pandemic. This suggests a genetic predisposition arising from a common ancestry. A 40 % HIV risk-reduction associated with Rh2 blood group was reported in a previous study by the same authors. The current study seeks to elucidate potential mechanisms for this risk reduction. Lymphocyte sub-populations and viral load measurements were achieved by routine diagnostic laboratory methods in 102 untreated HIV-1 infected patients. The results were compared across the categories of RH2 blood group. Carriage of the Rh2 antigen was associated with a higher proportion of T-cells (82±8% versus 75±10%, *P*=0.001), especially single-positive ($CD8^+$) cytotoxic T-cells (64±14 versus 54±13%, *P*=0.004). High absolute CD8 counts were more prevalent among the Rh2-positive than the Rh2-negative subjects (90 % versus 65 %, p=0.023). Among the Rh2-positive subjects, the increase in CD8 count paralleled the viral load in comparison to the Rh2-negatives (r^2=0.630, *P*<0.0001 versus r^2=0.148, *P*<0.001, respectively). The results suggest that Rh2 enhances the CD8 counts in an HIV infection, and its cells are known to play a vital role in immunity against HIV. This probably explains the protective role observed against HIV-1 infection.

Keywords: Rh2; HIV-prevalence, CD8 count, CD4 count, Viral load; African.

1. Introduction

HIV prevalence in Botswana has been ranked among the highest in the world (Weiser *et al.*, 2006). Many studies have revealed risk factors that have contributed to the spread of the virus, some of which include poverty, multiple sexual partners, alcohol and drug abuse, as well as the improper use of protective devices (Keetile, 2014). In some studies, the risk of infection in women was partly attributed to cultural norms that subject women to sexual abuse or assign them to a lower economic status (Shannon *et al*., 2012).

The high prevalence of HIV in Africa as well as those communities comprised of people of African descent, such as the Caribbean, strongly suggests a common genetic link. However, few such links have been alluded to. In at least two studies, the Duffy antigen receptor for chemokines was reported to promote HIV infection (He *et al*., 2008; Lachgar *et al*., 1998) among individuals of African extraction. However, these reports were strongly refuted by other investigators (Winkler *et al.*, 2009). In yet another study, the over-expression of the P^k blood group was reported to protect against HIV infection (Lund *et al*., 2009). However, this antigen is rare among Africans(Cooling, 2014). While it is generally accepted that host genetic factors play a role in immunity against the virus (Chatterjee, 2010), no specific hereditary factors have been advanced to explain the pandemic in Africa.

Although the function of many blood groups remains unknown, some reports have demonstrated that erythrocytes (red-blood cells) bind HIV, and such HIV becomes more efficiently transferred to CD4+(Beck *et al*., 2009; Beck *et al*., 2013; Garcia *et al*., 2012) cells. Erythrocyte antigens are therefore logical targets for erythrocyte-virus interactions. In a previous work by the authors of this study, it was observed that a 40 % risk reduction for HIV-1 infection in individuals of the blood group C (Rh2), while blood groups P_1 and Lu^b were associated with double and triple risks, respectively (Motswaledi *et al.*, 2016). Of an epidemiological importance was the observation that this protective blood group was very rare among Africans, while the risk-associated blood groups were much more common, which raises the question of whether this antigenic profile could have contributed to the peculiar susceptibility of this population to infection with HIV-1.

Blood group C is a component of the Rh blood group system. Antigens in this system are inherited as a block of genes in close proximity to each other on chromosome 1. This ensures that the genes are always inherited together. The RHD gene codes for the D antigen, while the RHCE gene carries a polymorphism that leads to the production of a range of RH phenotypes that include CE, Ce, cE or ce (Ripoche *et al*., 2004). The Rh gene products are organized on the erythrocyte membrane as a complex of proteins that include the Rh-associated glycoproteins (RhAG), LW, CD77, Duffy and CD47, which serve as ammonia transporters (Anstee and Tanner, 1993; Pourazar, 2007; Ripoche *et al*., 2004).

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The current study performed a viral load testing, CD4 and CD8 counts and compared the results among patients expressing or negative for Rh2. From these results and the review of some available literature, the current study sought to find out if the observed risk reduction for HIV infection could be corroborated by clinical laboratory data.

2. Materials and Methods

One hundred and two (102) HIV-1-infected (39 males and 63 females) and treatment-naïve individuals have been enrolled in this study. Since, no drug tests were done to verify their treatment-naïve status, all subjects with undetectable viral loads were excluded on the presumption that they could have been exposed to treatment.

Anonymized EDTA-anticoagulated samples from a central HIV testing laboratory in Gaborone have been used in this study. The ethical clearance was obtained from the University of Botswana's Office of Research and Development, Human Research and Development Committee of the Ministry of Health and Wellness, Gaborone District Health Management Team and the Research Ethics Committee of the Faculty of Health and Wellness Sciences at Cape Peninsula University of Technology. Individual consent was not needed since the study used anonymized residual samples (ISO, 2007).

The viral load was measured using the Cobas® Taqman 48 analyser (Pleasanton, CA., USA). CD3, CD4, CD8 and CD45 counts were obtained using the FacsCalibur ® flow cytometer (Becton-Dickinson, San Jose, CA, USA) and the CD3/CD8/CD45/CD4 Trucount panel. The samples were further phenotyped for the C antigen using specific anti-C and anti-c antibodies (Fortress Diagnostics, Antrim, UK). The reactivity of the antisera was confirmed with C/c-positive and negative cells selected from an antibody identification panel, Bio-Rad DiaPanel®, Lot 45241.88.1, (Cressier FR, Switzerland).

The results of the laboratory tests were interpreted in line with the reference ranges previously determined for the Botswana population (Mine *et al*.,2011). CD4 and CD8 counts from that study of normal individuals were also used to compare counts in the current study in a one-sample t-test.

The results were analyzed using the IBM SPSS version 24 statistical software. The independent t-test was used to compare means. Correlations were used to study the effect of blood RH2 on the relationship between CD8%, log viral load, CD8% and CD4%. Results were considered significant only if P<0.05.

3. Results

3.1. Results from Experimental Data

The mean viral load did not differ among individuals positive (n=21) or negative (n=81) for C (*P*=0.398). Representative dot plots for lymphocyte populations are shown in Figure 1.

In the untreated HIV-1-infected subjects, the CD4 count was significantly lower than that in the general uninfected population. On the contrary, the absolute CD8 count was significantly higher in the infected than in the uninfected subjects. The T-test results are shown in Table 1.

Individuals expressing the Rh2 antigen, 19/21 (90 %)) had a high absolute CD8 count compared to 52/80 (65 %) individuals who were Rh2-negative. A high CD8 count was therefore more associated with C-positive individuals than with the C-negative (*P*=0.023) population. The mean CD8 count between the two categories of Rh2, though higher in the C-positive group, did not reach statistical significance. However, the proportion of T-lymphocytes (CD3+) and cytotoxic (CD8+) T-cells was higher in the C-positive population as shown in Table 2.

Among C-positive subjects, the increase in CD8 counts paralleled the viral load more strongly than in the C-negatives. These results are shown in Figure 2.

Table 1. Comparison of CD4 and CD8 in untreated, HIV-infected individuals and normal controls.

Lymphocyte population	Mean in HIV-infected Subjects (n=101)	Mean in normal population (n=261)	*P*-value
CD4	307±208	859*	<0.0001
CD8	927±492	540*	<0.0001

*One sample (T-test) mean used from a previous study (Mine *et al.*, 2011).

Table 2. Comparison of mean Lymphocyte sub-populations in untreated HIV-infected patients

Lymphocyte Sub-population	C-Positive (Mean±sd) n = 21	C-Negative (Mean±sd) n = 80	*P*-value
CD3+ (%)	82±8	75.0±10	0.001
CD8+ (%)	64±14	54±13	0.004
CD8 Absolute count (/µL)	1058±444	893±500	0.171

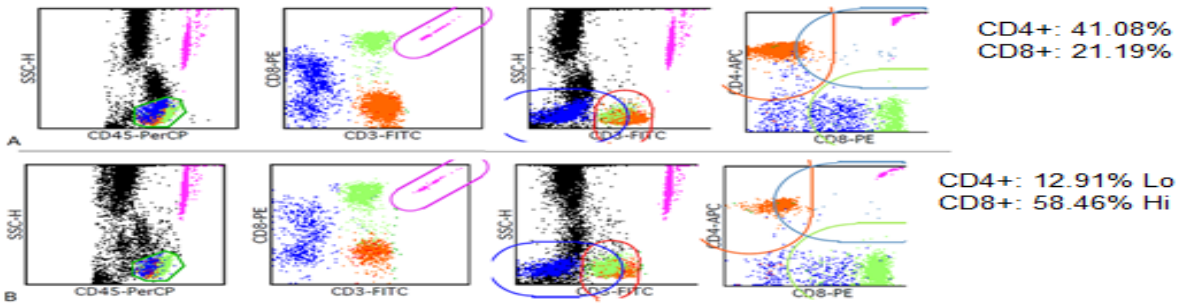

Figure 1. Lymphocytes were gated on the SSC-H vs CD45 and SSC-H vs CD3. A=normal control showing adequate CD4+ cells relative to CD8. The reverse is observed in the patient (B).

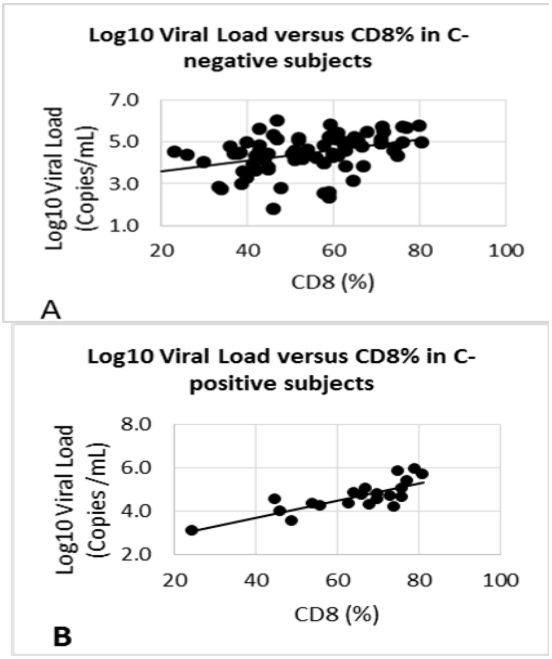

Figure 2. Relationship between viral load and CD8% in C-positive subjects shows strong positive correlation (r2=0.630, p<0.0001 versus r2=0.148, *P*<0.001).

3.2. Results from Country HIV Prevalence Data

To test this observation in real life, a literature search was undertaken to investigate HIV prevalence in countries where the frequencies of the Rh2 are known. Data was obtained for twenty countries or communities as shown in Table 3. Countries or communities with an Rh2 frequency less than 30 % consistently presented a high HIV prevalence (Pearson χ^2=20.0, *P*<0.0001).

Table 3. Comparison of Rh2 frequencies with HIV prevalence across geographical regions

Country/Community	Rh2 Frequency	HIV Prevalence
Botswana	29.70	21.90
South Africa (Blacks)	18.80	18.90
Nigeria	28.2	2.9*
Cote d'Ivoire	21.97	2.70
Caribbean	24.50	1.60**
Uganda	3.62	6.5***
Thailand	51.50	1.10
Ethiopia (Blacks)	41.18	1.10
South Africa (Asians)	99.53	1.00
Mali	58.8	1.00
Brazil	63	0.6
Mauritania	42.69	0.5
Argentina	62	0.4
Laos	60.30	0.30
Southern India	88.00	0.30
India	87.00	0.30
Northern India	84.76	0.26
Sudan	58.40	0.20
Netherlands	68.00	0.20
Iran	75.90	0.10
China	78.22	0.04

*5.8% in 2001. Some states at some point exceeded 8%. Nigeria carries the world's heaviest HIV burden(Gwaram, 2013).
**Prevalence rates range from 1-5% for different islands, depending on the reporting dates(Coggins, 2006; UNAIDS, 2016a). RH2 information for Curacao used here.
***Prevalence is down from 24.8% in 1994 (Kilian *et al*., 1999).

4. Discussion

4.1. Rh2, CD8 Parameters and Protection against HIV Infection

This study seeks to establish the mechanism of protection provided by carriage of Rh2 observed in a previous study. In this study, the expression of this antigen was associated with a higher proportion of cytotoxic (CD8+) T-cells (CTL), which corroborates protection against HIV. In C-positive subjects, the CD8 absolute count varied in a direct proportion to the viral load, suggesting a coordinated response to viremia. This correlation was much weaker in the C-negative group. Moreover, the proportion of individuals with high CD8 counts was greater in the C-positive group. All these observations suggest a role for this antigen in cytotoxic T-cell kinetics and function.

CTLs are important in the control of viremia in acute HIV-1 infections (Goonetilleke *et al*., 2009). They also correlate negatively with infection rates (Oxenius *et al*., 2004) and with good prognosis (Rinaldo *et al*., 1995). Moreover, they have been linked to the slower progression of disease in non-progressors by producing HIV-1-specific CTL responses (Alimonti *et al*., 2006; Betts *et al*., 2001) and increasing perforin production in the HIV-infected non-progressors (Migueles *et al*., 2002).

The enhancement of CTLs points to a mechanism involving major histocompatibility class I (MHC-I) response, in which a cell-mediated rather than humoral response is invoked, and this kind of response is critical for viral infections such as HIV. This was an unexpected finding for an erythrocyte antigen to be involved in an immune reaction. However, it is noted that erythrocytes and erythrocyte-bound platelets selectively bind infectious HIV virions (Beck *et al*., 2013). Furthermore, platelets have been shown to engage in direct MHC-I antigen presentation to CTLs (Chapman *et al*., 2012), a phenomenon that has also been documented in murine megakaryocytes (Zufferey *et al*., 2015). This study maintains that the Rh antigens co-localize on the membrane with CD47 (Ripoche *et al*., 2004), an activator of CTLs (Seiffert *et al*., 2001). Furthermore, both CD47 and Rh2 have been implicated as prognostic indicators in adenocarcinoma of the lungs (Schulze *et al*., 2018), noting that cell-mediated immunity is also critical for immunity against cancer cells.

CD47 also interacts with its ligands on monocytes and T-lymphocytes in signal-transducing events. In this regard, it binds to signal regulatory protein-α (SIRPα) in a high-affinity interaction that results in T-cell activation (Seiffert *et al*., 2001). The binding of CD47 to SIRPβ2 enhances adhesion of T-cells to antigen-presenting cells, and therefore enhances cell-mediated immunity (Piccio *et al*., 2005). In activated T cells, such as those occurring following the HIV infection, CD47 promotes Fas-mediated apoptosis (Manna *et al*., 2005). All these events work to eliminate HIV-infected CD4+cells, and therefore minimize the chances for an infection to be established.

4.2. Evidence from Empirical HIV Prevalence Data

To further investigate its potential role in the epidemiology of HIV worldwide, this study compared the frequency of the Rh2 antigen across populations with varying degrees of HIV prevalence. A thorough literature

search yielded Rh2 data from twenty countries. Invariably, countries with low frequency of Rh2 were consistently associated with the highest prevalence rates in their regions. In Southern Africa, antigen frequency data were available only for Botswana (Motswaledi et al., 2016) and South Africa (Tax *et al*., 2002). Cote d'Ivoire and Nigeria (Gwaram, 2013) likewise had the lowest antigen frequencies (Bogui *et al*., 2014) and the highest HIV prevalence in West Africa (UNAIDS, 2016a). In contrast, other African countries with higher antigen frequencies had low HIV-prevalence rates comparable to or slightly above those in other non-African countries, such as Argentina (Cotorruelo *et al*., 2008), Brazil (Guelsin *et al*., 2011), Thailand (Nathalang *et al*., 2001), Laos (Keokhamphoui *et al*., 2012), India (Makroo *et al*, 2013), Netherlands (Tax *et al*., 2002), Iran (Shokouhi Shoormasti *et al*., 2011), and China (Ma *et al*., 2018), where the Rh2 frequency is higher than 40 %. These African countries include Mali (Ba, *et al*., 2015), Mauritania (Hamed *et al*., 2013), Ethiopia (Tax *et al*., 2002), Sudan (Elfadni *et al*., 2014), and among South Africans of Asian origin (Tax *et al*., 2002).

Rh2 data for Caribbean Islands were not readily available, except for Curacao. The Caribbean Islands represent the second highest HIV-prevalence outside Africa (UNAIDS, 2016b). Apparently, a significant proportion of this population originates from Africa, and the Rh2 antigenic profile performed in Curacao (Tax *et al*., 2002) is similar to that of the African countries where high HIV rates are found. This study proposes that the Rh2 antigen may be important for HIV immunology and probably explains the genetic basis for the geographical distribution of the pandemic.

5. Conclusion

The low frequency of the Rh2 antigen in Botswana and other countries with similar frequencies may have contributed to the rapid spread of HIV-1 among the populations of these countries. Results also suggest an immunological role of an Rh-system antigen, unreported hitherto. However, the current study has been limited in that it has not measured specific CD8 immune responses such as the interferon gamma response to specific HIV-1 peptides in C-positive and negative individuals. Furthermore, other co-variables that are known to affect the risk of infection were not considered in this cross-sectional study.

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References

Alimonti J B, Kimani J, Matu L, Wachihi C, Kaul R, Plummer F A and Fowke KR. 2006. Characterization of CD8+ T-cell responses in HIV-1-exposed seronegative commercial sex workers from Nairobi, Kenya. *Immunol Cell Biol*, ***84*(5)**: 482-485.

Anstee D J and Tanner M J. 1993. Biochemical aspects of the blood group Rh (rhesus) antigens. *Baillieres Clin Haematol*, ***6*(2)**: 401-422.

Ba A, Beley S, Chiaroni J, Bailly P and Silvy M. 2015. RH diversity in Mali: characterization of a new haplotype RHD*DIVa/RHCE*ceTI(D2). *Transfusion*, ***55*(6 Pt 2)**, 1423-1431.

Beck Z, Brown B K, Wieczorek L, Peachman K K, Matyas G R, Polonis V R, et al. 2009. Human erythrocytes selectively bind and enrich infectious HIV-1 virions. *PLoS ONE*, *4*(12).

Beck, Z., Jagodzinski, L. L., Eller, M. A., Thelian, D., Matyas, G. R., Kunz, A. N., et al. 2013. Platelets and erythrocyte-bound platelets bind infectious HIV-1 in plasma of chronically infected patients. *PLoS ONE*, ***8*(11)**, e81002. doi: 10.1371/journal.pone.0081002

Betts, M. R., Ambrozak, D. R., Douek, D. C., Bonhoeffer, S., Brenchley, J. M., et al. 2001. Analysis of total human immunodeficiency virus (HIV)-specific CD4+ and CD8+ T-cell responses: relationship to viral load in untreated HIV infection. *J Virol*, ***75*(24)**: 11983-11991.

Bogui, L. S., Dembele, B., Sekongo, Y., Abisse, S., Konaté, S., & Sombo, M. 2014. Phenotypic profile of Rh and Kell blood group systems among blood donors in Cote d'Ivoire, West Africa. *J Blood Transfusion*. doi:10.1155/2014/309817.

Chapman, L. M., Aggrey, A. A., Field, D. J., Srivastava, K., Ture, S., Yui, K., et al. 2012. Platelets present antigen in the context of MHC class I. *J Immunol*, *189*(2), 916-923.

Chatterjee, K. (2010). Host genetic factors in susceptibility to HIV-1 infection and progression to AIDS. *J Genet*, *89*(1), 109-116.

Coggins, P. C. 2006. *Cultural Issues for HIV/AIDS Clients from the Caribbean:Myths and Realities.* Paper presented at the Annual Grantee meeting of the Federal Government's Center for Substance Abuse Treatment Targeted Capacity Annual Expansion HIV and HIV Outreach Washington, D.C. http://www.guyanajournal.com/HIV-AIDS_Caribbean.html

Cooling L. 2014. P Blood Groups/ Glob collection. *In* Fung M K, Grossman B J, Hillyer C D and Westhoff, C M (Eds.), **Technical Manual** (18 ed.,). Bethesda, Maryland: American Association of Blood Banks.pp 309.

Cotorruelo, C M, Fiori, S V, Borrás, S E G, Racca, L L, Biondi, C S and Racca, A L. 2008. Distribution of the FYB ES and RHCE*ce(733C>G) alleles in an Argentinean population: Implications for transfusion medicine. *BMC Medical Genetics*, **9(1)**, 40. doi: 10.1186/1471-2350-9-40.

Elfadni, M, Yassin, H, Abushama, H, Khalid, E and Khalil, E. 2014. Rh Blood group system phenotypes, haplotypes and probable genotypes among major sudanese tribes. *Int J Curr Res*, **6(8)**, 7862-7866.

Garcia, M N, dos Ramos Farias, M S, Fazzi L, Grasso, D, Rabinovich, R D and Avila, M M. 2012. Presence of IgG anti-gp160/120 antibodies confers higher HIV capture capacity to erythrocytes from HIV-positive individuals. *PLoS ONE*, **7(9)**, e45808. doi: 10.1371/journal.pone.0045808

Goonetilleke, N, Liu, M K, Salazar-Gonzalez, J F, Ferrari, G, Giorgi, E, Ganusov, V. V., et al. 2009. The first T cell response to transmitted/founder virus contributes to the control of acute viremia in HIV-1 infection. *J Exp Med*, **206(6)**, 1253-1272.

Guelsin, G A S, Sell, A M, Castilho, L, Masaki, V L, de Melo, F C, Hashimoto, M N, et al. (2011). Genetic polymorphisms of Rh, Kell, Duffy and Kidd systems in a population from the State of Paraná, southern Brazil. *Revista Brasileira de Hematologia e Hemoterapia*, ***33*(1)**, 21-25. doi: 10.5581/1516-8484.20110009.

Gwaram, B. 2013. Prevalence of Rh Phenotypes among Blood Donors in Kano, Nigeria. *J Med Trop*, ***15*(1)**, 37-39.

Hamed, C T, Bollahi, M A, Abdelhamid, I, Sow, A, Sidi, A M, Habti, N, et al. (2013). Distribution of Rhesus and Kell blood group frequencies in the Mauritanian population. *Blood Transfusion*, ***11*(1)**, 154-155. doi: 10.2450/2012.0022-12

He, W, Neil, S, Kulkarni, H, Wright , E and Agan, B K. 2008. Duffy antigen receptor for chemokines mediates trans-infection of HIV-1 from red blood cells to target cells and affects HIV-AIDS susceptibility. *Cell Host Microbe*, **4(1)**, 52–62.

International Organization for Standardization. (2007). Ethics in laboratory medicine *ISO:15189*. (pp. Annexture C9). Geneva.

Keetile, M. 2014. High-risk behaviors among adult men and women in Botswana: Implications for HIV/AIDS prevention efforts. *Sahara J*, ***11*(1)**, 158-166. doi: 10.1080/ 17290376.2014.960948

Keokhamphoui, C, Urwijitaroon, Y, Kongphaly, D and Thammavong, T. 2012. Blood group antigen distribution in Lao blood donors. *Immunohematol.*, ***28*(4)**, 132-136.

Kilian, A H, Gregson, S, Ndyanabangi, B, Walusaga, K, Kipp, W, Sahlmüller, G.,et al. 1999. Reductions in risk behaviour provide the most consistent explanation for declining HIV-1 prevalence in Uganda. *AIDS*, ***13*(3)**, 391-398.

Lachgar, A, Jaureguiberry, G, Le Buenac, H, Bizzini, B, Zagury, J F, Rappaport, J., et al. 1998. Binding of HIV-1 to RBCs involves the Duffy antigen receptors for chemokines (DARC). *Biomed Pharmacother*, ***52*(10)**, 436-439.

Lund, N, Olsson, M L, Ramkumar, S, Sakac, D, Yahalom, V, Levene, C, et al. 2009. The human Pk histo-blood group antigen provides protection against HIV-1 infection. *Blood Rev*, ***113*(20)**, 4980-4991.

Ma, T, Yang, J, Song, Y, Wang, W, Xie, X, Chen, P, et al. 2018. [Distribution of Rh blood group in 51 283 cases of inpatients and voluntary blood donors]. *Xi Bao Yu Fen Zi Mian Yi Xue Za Zhi*, ***34*(1)**, 70-74.

Makroo, R N, Bhatia, A, Gupta, R and Phillip, J. 2013. Prevalence of Rh, Duffy, Kell, Kidd and MNSs blood group antigens in the Indian blood donor population. *Indian J Med Res*, **137**, 2521-2526.

Manna, P P, Dimitry, J, Oldenborg, P A and Frazier, W A. 2005. CD47 augments Fas/CD95-mediated apoptosis. *J Biol Chem*, ***280* (33)**, 29637-29644. doi: 10.1074/jbc.M500922200

Migueles, S A, Laborico, A C, Shupert, W L, Sabbaghian, M S, Rabin, R, Hallahan, C W, et al. 2002. HIV-specific CD8+ T cell proliferation is coupled to perforin expression and is maintained in nonprogressors. *Nature immunol*, ***3*(11)**, 1061-1068.

Mine, M, Moyo, S, Stevens, P, Michael, K, Novitsky, V, Makhaola, K, et al. 2011. Immunohaematological reference values for HIV-negative healthy adults in Botswana. *Afr J Lab Med*, ***1*(1)**, 5-11.

Motswaledi, M S, Kasvosve, I and Oguntibeju, O O. 2016. Blood Group Antigens C, Lub and P1 may have a role in HIV infection in Africans. *PLoS ONE*, *11*(2), e0149883. doi: .10.1371/ journal.pone.0149883

Nathalang, O, Kuvanont, S, Punyaprasiddhi, P, Tasaniyanonda, C, and Sriphaisal, T. 2001. A preliminary study of the distribution of blood group systems in Thai blood donors determined by the gel test. *Southeast Asian J Trop Med Public Health*, ***32*(1)**, 204-207.

Oxenius, A, Price, D A, Hersberger, M, Schlaepfer, E, Weber, R, Weber, M., et al. 2004. HIV-specific cellular immune response is inversely correlated with disease progression as defined by decline of CD4+ T cells in relation to HIV RNA load. *J Infect Dis*, ***189*(7)**, 1199-1208.

Piccio, L., Vermi, W., Boles, K. S., Fuchs, A., Strader, C. A., Facchetti, F., . . . Colonna, M. 2005. Adhesion of human T cells to antigen-presenting cells through SIRPβ2-CD47 interaction costimulates T-cell proliferation. *Blood*, ***105*(6)**, 2421-2427. doi: 10.1182/blood-2004-07-2823

Pourazar, A. 2007. Red cell antigens: Structure and function. *Asian J Transfus Sci*, **1(1)**, 24-32. doi: 10.4103/0973-6247.28069

Rinaldo, C., Huang, X.-L., Fan, Z., Ding, M., Beltz, L., Logar, A.,et al. 1995. High levels of anti-human immunodeficiency virus type 1 (HIV-1) memory cytotoxic T-lymphocyte activity and low viral load are associated with lack of disease in HIV-1-infected long-term nonprogressors. *J Virol*, ***69*(9)**, 5838-5842.

Ripoche, P, Bertrand, O, Gane, P, Birkenmeier, C, Colin, Y and Cartron, J.-P. 2004. Human Rhesus-associated glycoprotein mediates facilitated transport of NH3 into red blood cells. *PNAS USA*, ***101*(49)**, 17222-17227. doi: 10.1073/pnas.0403704101

Schulze, A B, Schmidt, L H, Baie, L, Heitkotter, B, Kuemmel, A, Mohr, M.,et al. 2018. Rhesus CE expression on patient red blood cells is an independent prognostic factor for adenocarcinoma of the lung. *Clin Respir J*, ***12*(3)**, 1106-1117. doi: 10.1111/crj.12638

Seiffert, M., Brossart, P., Cant, C., Cella, M., Colonna, M., Brugger, W., et al. 2001. Signal-regulatory protein α (SIRPα) but not SIRPβ is involved in T-cell activation, binds to CD47 with high affinity, and is expressed on immature CD34⁺CD38[−]hematopoietic cells. *Blood*, ***97*(9)**: 2741-2749. doi: 10.1182/blood.V97.9.2741

Shannon, K., Leiter, K., Phaladze, N., Hlanze, Z., Tsai, A. C., Heisler, M.,et al. 2012. Gender inequity norms are associated with increased male-perpetrated rape and sexual risks for HIV infection in Botswana and Swaziland. *PLoS ONE*, ***7*(1)**: e28739. doi: 10.1371/journal.pone.0028739

Shokouhi Shoormasti, R, Azimdoost, A, Saghafi, S, Movahhedi, M., Haghi Ashtiani, M. T., Pourpak, Z., et al. 2011. Normal range determination of lymphocytes subsets in normal adults in Iran. *Iran J Allergy Asthma Immunol*, ***10*(4):** 295-298. doi: 010.04/ijaai.295298

Tax, M G, van der Schoot, C E, van Doorn, R, Douglas-Berger, L, van Rhenen, D J and Maaskant-vanWijk, P A. 2002. RHC and RHc genotyping in different ethnic groups. *Transfusion*, ***42*(5)**: 634-644.

UNAIDS. (2016a). HIV Prevalence. Retrieved 19th April 2018, from http://aidsinfo.unaids.org/

UNAIDS. (2016b). UNDAIDS fact sheet 06-Carribean. from http://www.who.int/hiv/mediacentre/200605-FS_Caribbean_en.pdf

Weiser, S. D., Leiter, K., Heisler, M., McFarland, W., Percy-de Korte, F., DeMonner, S. M., et al. (2006). A population-based study on alcohol and high-risk sexual behaviors in Botswana. *PLoS Med*, ***3*(10)**, e392. doi: 10.1371/journal.pmed.0030392

Winkler, C A, An, P, Johnson, R, Nelson, G W and Kirk, G. 2009. Expression of Duffy antigen receptor for chemokines (DARC) has no effect on HIV-1 acquisition or progression to AIDS in African Americans. *Cell Host Microbe*, ***5*(5):** 411-413.

Zufferey, A, Speck, E R, Guo, L, Aslam, R, Kapur, R and Semple, J W. 2015. Murine bone marrow-derived megakaryocytes are capable of antigen cross-presentation on major histocompatibility class (MHC) I molecules. *Blood*, ***126*(23):** 3465-3465.

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Detection of ESBL and MBL in *Acinetobacter* spp. and Their Plasmid Profile Analysis

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Abstract

The prevalence of extended spectrum β-lactamases (ESBLs), metalloβ-lactamases (MBL), and plasmid-mediated resistance is a severe threat to current lactam therapy leading to treatment failure. Therefore, in this research, an investigation has been conducted to study the presence of ESBL and MBL genes in *Acinetobacter* spp. and their relationship to antimicrobial resistance. Fifty *Acinetobacter* spp. isolates were collected from both pediatric and adult patients at Sungai Buloh Hospital, Kuala Lumpur, Malaysia. The Double Disk Synergy Test (DDST) and the E-test have been used to detect ESBL and MBL production. The plasmid was extracted using the Close and Rodriguez (1982) method with modification in addition to the QIAprep Spin Kit. Among the fifty *Acinetobacter* isolates, eleven (22%) were ESBL-positive, while none of the strains produced MBLs. Fourteen isolates were positive for a plasmid with an occurrence rate (POR) of 28 %. These fourteen isolates appeared to harbor one or more plasmids with a maximum of four plasmids. Four isolates had single plasmids, and five carried four plasmids, while four and one isolates had two and one plasmids respectively. The sizes of the plasmid DNA were found to range between 1.8 kb and 14 kb. This study emphasizes the problematic raising frequency of ESBL, MBL, and plasmid-mediated resistance. This awareness highlights an urgent need to prevent and control such bacteria.

Keywords: *Acinetobacter* spp., ESBL; MBL, Malaysia, Plasmid profile.

1. Introduction

Acinetobacter spp. has appeared as a significant wide-spread nosocomial pathogen implicated with a fatality. They are aerobic, non-fermentative, and gram-negative bacilli (Howard *et al.*, 2012). *Acinetobacter* spp. causes many diseases like pneumonia, septicemia, wound sepsis, urinary tract infections, endocarditis, meningitis, and some clinical diseases including blood stream infections (BSI) (Zurawski *et al.*, 2012). Over the last decades, multidrug-resistant (MDR) clinical isolates of *Acinetobacter* spp. have been reported as a consequence of the extensive use of potent broad-spectrum antimicrobial agents in hospitals throughout the world (Towner, 2009). Furthermore, *Acinetobacter* spp. contaminates different types of commercial food such as meat, fruits, vegetables and various types of livestock. *Acinetobacter* spp. associated with this contaminated food can multiply in the digestive tract of humans through environmental routes of transmission (Wong *et al.*, 2017; Zhang *et al.*, 2013). *A. baumannii* is the most virulent of all the species based on clinical data by statistical analysis and studies of animal models (Chusri *et al.*, 2014). The identification of ESBL producing *Acinetobacter* spp. is essential due to its multidrug resistance property (Bush, 2010). Possible risk factors related to the development of colonization or infection of hospitalized patients with *Acinetobacter* spp. extended the length of hospital stay, the severity of the disease, offensive procedures, and treatment (Ibrahimagic *et al.*, 2017). Studies showed that the environmental strains of *Acinetobacter* spp. Possess antibiotic resistance mechanisms (Al Atrouni *et al.*, 2016). The emerging antibiotic resistance is a serious global concern, resulting in treatment failures and increasing healthcare costs. The production of β-lactamase is the most common cause of bacterial resistance to beta-lactam antibiotics. Metallo-β-lactamases is a set of enzymes that catalyze the hydrolysis of carbapenems and a broad range of β-lactam drugs (Palzkill, 2013). The antibiotic resistance by ESBLs and MBLs gene production is increasing significantly in the clinical isolates of *Acinetobacter* spp. around the world. Studies have shown that bacteria producing ESBLs or MBLs are associated with higher mortality and morbidity (Delgado-Valverde *et al.*, 2013; Oberoi *et al.*, 2013). Extended-spectrum beta-lactamases (ESBLs) confer resistance to various groups of antibiotics (penicillins, cephalosporins, and aztreonam) (Aqel *et al.*, 2014). ESBLs are mostly encoded by plasmid- encoding resistance genes for a variety of antimicrobial agents (Cheaito and Matar, 2014). MBLs gene encoded by transposons transfers large transferable plasmids among different bacterial species and genera (Al-Marjani *et al.*, 2013). Plasmids are extra-chromosomal, circular, double-stranded DNA molecules,

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and play an essential role in bacterial multi-drug resistance. Plasmid-mediated antibiotic resistance is common in *Acinetobacter* spp. and plasmid profile analysis examines the total bacterial plasmid content (Nazmul MHM *et al.*, 2016). In Asia, one of the three most common causes of bacteremia and nosocomial pneumonia is due to *Acinetobacter* spp. (Chung *et al.*, 2011; Kuo *et al.*, 2012). There are approximately one million (range, 600,000 to 1,400,000) cases globally per year (Spellberg and Rex, 2013). Therefore, the outcome of this study might highly be effective for an early recognition of infections, the choice of antibiotics, limiting clinical use of antibiotics, the failure treatment of broad-spectrum β-lactams, and for the control of the prevalence of antibiotic-resistance mechanism, which is necessary to prevent the spread of multi-drug resistance throughout the world.

2. Materials and Methods

For this study, fifty *Acinetobacter* spp. isolates were obtained from the blood, skin, pus, respiratory secretions, urine, and sputum of both pediatric as well as adult patients of Sungai Buloh Hospital, Kuala Lumpur, Malaysia. Furthermore, the presence of both ESBL and MBL genes were confirmed by Double Disk Synergy Test (DDST) and E-test. In DDST**,** discs of third-generation cephalosporins and augmentin were kept 30mm separately from center to center on inoculated Muller-Hinton Agar (MHA). A perfect extension of the edge of the inhibition zone of cephalosporin to the augmentin disc was interpreted as positive for the ESBL production.

The phenotypic confirmatory test was evaluated for ESBL-producing *Acinetobacter* spp. Briefly, a 0.5 MacFarland's suspension of each strain was spread on a Muller – Hinton agar (MHA) plate. Ceftazidime (30µg) and ceftazidime / clavulanic acid (30µg/ 10µg) discs were placed aseptically on the agar plate. The distance was 15mm between the two discs (edge to edge), and the culture was incubated at 37°C overnight. Based on The Clinical and Laboratory Standards Institute (CLSI), ESBL production was confirmed when the inhibition zone increased to more than or equal to 5mm surrounding the combined antimicrobial agent compared to the single antimicrobial agent (CLSI, 2017). The increase in the zone diameter is due to the inhibition of the β-lactamase by the clavulanic acid (Thomson *et al.*, 2018). The procedures for the E-test were followed according to the manufacturer's instructions.

Close and Rodriguez's (1982) method was used with modification to detect plasmids. The manufacturer's instructions were followed to extract plasmid DNA using extraction kit (QIAprep spin kit, USA). The Supercoil DNA marker was used to estimate the plasmid size. It was used in the electrophoresis gel each time along with the plasmids as a molecular weight (range, 2.0kb to 10.0kb) marker, bought from Promega, USA.

3. Results

About fifty isolates of *Acinetobacter* spp. were identified by conventional bacteriological tests. Ten different types of commonly prescribed antibiotics have been used in this study to evaluate and compare the antibiotic resistance rate between the isolates.

All the clinical isolates (fifty) of *Acinetobacter* spp. showed a high frequency of resistance to ampicillin, meropenem, imipenem, and ceftazidime with a resistance rate of 92 %, 82 %, 74 %, and 74 % respectively (Table 1). Some of the isolates showed moderate resistance to the antibiotics tazobactam-piperacillin, ciprofloxacin, piperacillin, gentamicin, amikacin, and cefoperazone (68 %, 64 %, 54 %, 56 %, 56 %, and 64 % respectively).

ESBL-producing strains may play an essential role in the early recognition of infection control according to age and sex. Most of the ESBL-producing isolates were found to be in the age group of 41-50, and >50 years, while none of the ESBL-producing strainswere belong to the age group 11-20 years (0.00%).

The frequency of ESBL-producing isolates from different types of clinical samples is shown in Figure 1. Aseptic precautions were taken during the isolation of *Acinetobacter* spp. Among the clinical samples, urine (38 %) was the frequent source followed by blood (26 %), pus (14 %), skin (10 %), respiratory secretions (6 %), and sputum (6 %) respectively. The highest percentage of ESBL production was detected in the urine (12 %) followed by blood (6 %), sputum, and pus 2 % respectively (Figure 1). Furthermore, strains from respiratory secretions and skin did not produce any ESBL.

Table 1. Antibiotype of isolated *Acinetobacter* spp.

SL	Total isolates (50)	Antibiotyping										ESBL (+)	MBL (+)
		TPZ	CAZ	CFP	CIP	AK	MEM	IPM	PRL	AMP	CN		
1	Number of resistance	34	37	32	32	28	41	37	27	46	28	11	0
2	Percentage of resistance	68	74	64	64	56	82	74	54	92	56	22	0

Tazobactam 10/ Piperacillin 75-TZP, Ceftazidime-CAZ, Cefoperazone-CFP, Ciprofloxacin-CIP, Amikacin-AK, Meropenem-MEM, Imipenem-IPM, Piperacillin-PRL, Ampicillin-AMP, Gentamicin-CN.

Acinetobacter spp. was tested to detect the ESBL production and the occurrence rate of ESBL-producing strains. Among the fifty isolates of *Acinetobacter* spp., 22 % were found to produce ESBL in the phenotypic test and the E-test. The percentage of ESBL-negative isolates (78 %) were observed in both the phenotypic analysis and the E-test are shown in table 2. None of the strains was found to produce MBLs.

Table 2. Detection of ESBL-producing strains using the phenotypic test and the E-test.

	Total Number of the organism	Phenotypic test for ESBL		E-test for ESBL	
Organism		Positive	Negative	Positive	Negative
Acinetobacter spp.	50	11(22%)	39(78%)	11 (22%)	39(78%)

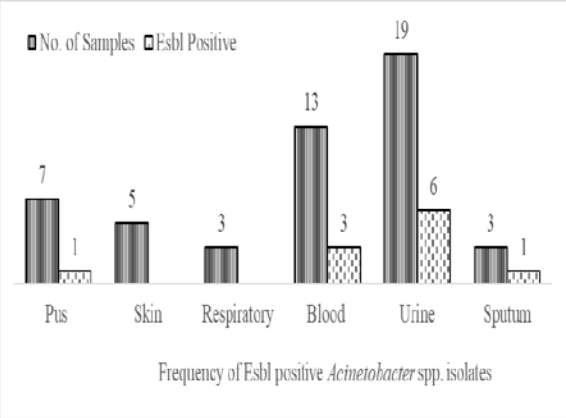

Figure 1. Frequency of ESBL-producing isolates from different types of samples.

ESBL-positive isolates were confirmed by the Double Disk Synergy Test (DDST). The inhibition zone between disks of AMC30 and CAZ30 were superimposed, and this synergy (Figure 2) confirmed the presence of ESBL production by the specific isolates. The occurrence rate of ESBL-producing strains of *Acinetobacter* spp. is shown in Figure1.

However, among the fifty isolates of *Acinetobacter* spp., none of the isolates produced MBL.

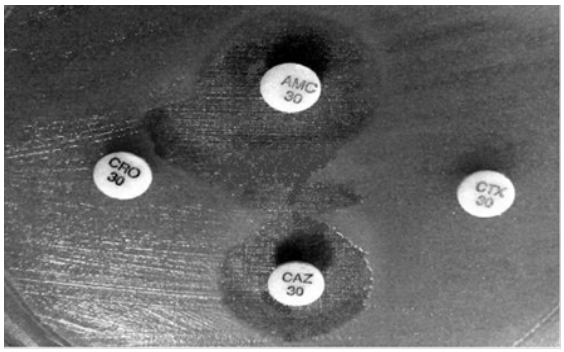

Figure 2. Double Disk Synergy Test showing ESBL production

The Close and Rodrigue method was performed for plasmid profile analysis, and the commercial extraction kit was used to detect plasmid DNA. The similarity of Plasmid DNA was found in both described methods. In Figure 3, the left lane was done by the Close and Rodrigue method, and the right lane was done by the commercial extraction kit (QIAprep spin kit) for a better observation. The presence of both chromosomes and plasmids was observed in *Acinetobacter* spp. under 0.7 % gel electrophoresis (Figure3).

Out of the fifty *Acinetobacter* spp., fourteen isolates were found to harbor plasmid. Few strains were found to carry a maximum of four plasmids (Figure 4). The size of the plasmids varied from 1.8 to 14kb (1.8, 2.2, 3.4, 4.6, 5, 8 and 14 kb) under 0.7 % gel electrophoresis. Plasmid occurrence rate (POR) was 28 % (fourteen isolates) among the studied isolates, and thirty-six (72 %) strains did not harbor any plasmid DNA (Table 3).

Table 3. Plasmid occurrence rate (POR) in *Acinetobacter* spp. isolates.

No. of Plasmid DNA	Isolates No.	POR%
0	36	72
1	3	6
2	4	8
3	3	6
4	4	8
5	0	0

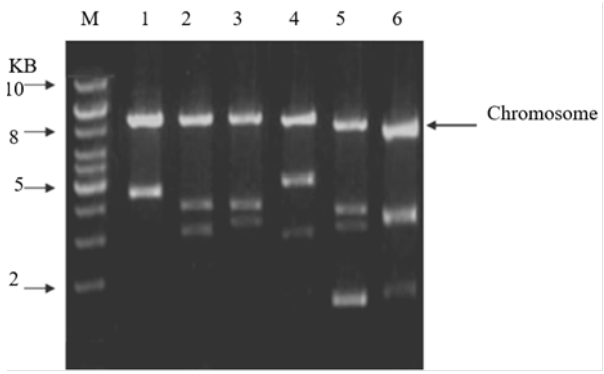

Figure 3. Comparison of the two methods used in this study

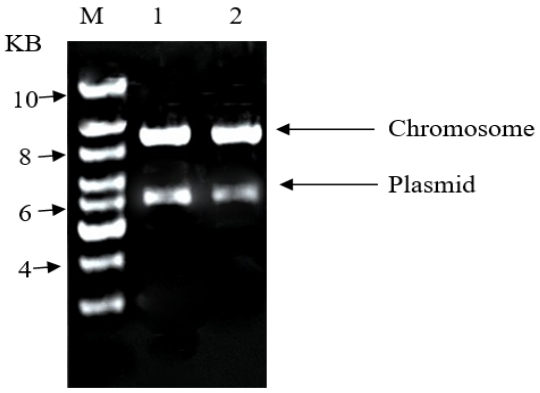

Figure 4. Agarose (0.7 %) gel electrophoresis of plasmid DNA

4. Discussion

The prospective sources of ESBL associated with an infection, its spread, and colonization of humans have been studied considerably over the past few years (Walther *et al.*, 2018). The highest occurrence of antibiotic resistance in this study was exhibited by the carbapenems and cephalosporins groups of antibiotics (Table 1). This is in agreement with another recent study in Pakistan (Sohail *et al.*, 2016). The frequency of ESBL-producing isolates of *Acinetobacter* spp. from different types of clinical sources is shown in Figure 1. In this present study, urine showed the highest number (37.58 %) as an ESBL producer compared with other clinical sources of isolates; this finding is an agreement with another recent study in 2017 (89 %) (Park and Kim, 2017). The DDST is one of the most common and confirmative tests to detect ESBL production based on the synergistic effects of clavulanate and ceftazidime in the presence of clavulanic acid (Figure 2). Both DDST and E-test showed similar results regarding the ESBL production (Table 2). The production of ESBL confers resistance at the various levels to expanded-

spectrum cephalosporins (cefotaxime and ceftazidime) and aztreonam, but typically not to the cephamycins and carbapenems (Shaikh *et al.*, 2015). Recently, a substantial increase in the occurrences of ESBL-related infections has noted been throughout the globe.

This study showed that the ESBL-producing *Acinetobacter* spp. were 11 out of 50 (22 %) isolates by both phenotypic and E-test. The outcome of the current research has a similarity with another study by Owlia et al., (Owlia *et al.*, 2012) where it was reported that 21 % of *Acinetobacter* spp. produced ESBL. However recent studies done in Bangladesh reported a lower rate (9.09 %), five out of fifity-five *Acinetobacter* spp. isolates were ESBL-positive (Paul *et al.*, 2017) and 7.24 % of ESBL production among *Acinetobacter* spp. Isolates (Dhillon and Clark, 2012). Similarly, another study performed in Saudi Arabia reported that 8.1 % of *Acinetobacter* strains isolated from burns' patients were ESBL-producers (Al-Tawfiq and Anani, 2009). A number of studies showed a higher occurrence for ESBL production, 44 % out of 147 *Acinetobacter* spp. in 2010 (Hashemizadeh *et al.*, 2010), 46 % in 2001 (Vahaboglu *et al.*, 2001), 23 % by Soudeiha *et al.*, (Soudeiha *et al.*, 2018), 21.74 % in 2018 (Hafiz *et al.*, 2018) and 54.6 % (Yong *et al.*, 2003). In addition, Pournajaf and his colleagues in 2018 showed 78 % of isolates to produce ESBL using Double Disk Synergy Test, and 83.5 % of the isolates to produce ESBL using E-test (Pournajaf *et al.*, 2018); these findings are closer to those by a another study reporting 88 % (88/100) of ESBL production (Goyal *et al.*, 2018). There was a significant association between ESBL production based on sex and age in this present study. The highest frequency of ESBL was reported in 18.18 % of the isolates in the age group of 21-40 years in male and 36.37 % in females. The prevalence of ESBL production was significantly different between males and females in all age groups and was observed to be 27.27 % and 72.73 % respectively.

In the present study, after screening among all the *Acinetobacter* spp.isolates, none of the isolates produced the MBL gene, and this is an agreement with a recent study in 2014 (Al-Agamy *et al.*, 2014). But a number of studies showed MBL productions, 39 % (42 out of 108) isolates in 2012 (Owlia *et al.*, 2012), 10 % in 2007 (Pino *et al.*, 2007), and fifty strains (96 %) were found to be MBL-positive by the modified Hodge test in 2015 (Aksoy *et al.*, 2015). However, the presence of MBL in *Acinetobacter* spp. was less common (Irfan *et al.*, 2011; Shoja *et al.*, 2017).

In the nosocomial pathogens, the transfer of antibiotic resistant genes causes complications in patients' treatment (Beige *et al.*, 2015). In recent years, several protocols have developed to simplify the isolation of plasmid DNA. Most of these rapid procedures are invariably contaminated with chromosomal DNA. The detection of plasmids depends on the adequately used methods (Nazmul MHM *et al.*, 2016). For the plasmid profile analysis, the Close and Rodriguez method (1982) with modification and the plasmid extraction kit (QIAprep spin kit) were used to extract and purify plasmid DNA from the bacterial isolates. The most purified plasmids without contamination with genomic DNA was detected by the plasmid DNA extraction kit (QIAprep spin kit) compared to the Close and Rodriguez method. Plasmid DNA extraction kit could even purify small-sized plasmids. The reproducible recovery of all plasmid DNA was seen by the plasmid DNA extraction kit in *Acinetobacter* spp.

In the present study, the supercoil DNA marker (molecular weight markers) was used to estimate the plasmid size of *Acinetobacter* spp.in each gel. Plasmids were detected from fourteen strains, and the overall sizes of the plasmid DNA ranged from the lowest 1.8 kb to the highest 14 kb, while another study (Sadeghifard *et al.*, 2011) observed the sizes of the plasmid DNA to range from the lowest 1kb to the highest 21kb. A survey in KSA showed that five *A. baumannii* isolates harboured plasmids among nineteen isolates which were not less than 2.71kbp in molecular weight (Selim and Hagag, 2013). The results of the current study are also in agreement with another recent study in Malaysia which showed that 40 % of the isolates harbored plasmid DNA ranging from 1.8kb to 8kb (Nazmul MHM *et al.*, 2016). Cameranesi *et al*. reported 9kbp to slightly more than 23 kbp (Cameranesi *et al.*, 2018) plasmids, whereas a pK50a (79.6 kb) plasmid was found by Wibberg *et al.*, 2018. Besides, according to a study in 2014, plasmids were found in eighty out of eighty-eight isolates (90.9 %) with one being of a 7.30kbp molecular weight (Ali *et al.*, 2014). Some researchers have reported a much lower rate of plasmid detection 42 % of the tested strains (Koeleman *et al.*, 2001), seventeen isolates (34 %) carried plasmids with molecular sizes of > 20kbp (Eftekhar *et al.*, 2018), and the isolates contained a molecular weight of a plasmid ranging between 2 to > 25kb (Saranathan *et al.*, 2014). Thirty-six isolates (72 %) did not carry any plasmids in the current study which is in agreement with other studies in 2016 (60 %) (Nazmul MHM *et al.*, 2016) and 2018 (11 %); the isolates did not carry any plasmid (Salto *et al.*, 2018).

These fourteen isolates appear to harbor one or more plasmids with the maximum number of four plasmids. Various plasmid profiles were determined where four isolates owned single plasmids, and five strains carried four plasmids, while four and one isolates had two and one plasmids respectively. Plasmid values are essential for antibiotic resistance studies as they can specify or identify the plasmid-mediated resistance gene in addition to in typing different bacterial strains. The total plasmid occurrence rate (POR) was (28 %) when using the Close and Rodriguez method with modification (1982) and the QIAprep spin kit. This finding is in agreement with Saranathan and his colleagues who detected fifty isolates (94·5 %) in 2014 (Saranathan *et al.*, 2014), and plasmids were found in 107 out of the 112 (95.5 %) strains (Sadeghifard *et al.*, 2011).

A number of studies around the world have shown the involvement of plasmids in multidrug resistance (Botts *et al.*, 2017; Mathers *et al.*, 2015; Porse *et al.*, 2016; Weber *et al.*, 2015). Among the gram-negative bacteria, plasmids-encoding genes are transferred to the variety of microbial agents by transmission, and this can lead to serious public health hazards. In addition, the β-lactam resistance genes in the plasmid are supported by plasmid DNA analysis. Some of these genes may characterize the chromosome-based mechanisms of resistance. According to the present study, the involvement of ESBL, MBL, and plasmid association may play an essential role in resistance. The differently sized plasmids found in the current investigation might be involved in multidrug resistance. Further studies are required to find out the association of

these plasmids in multidrug resistance and β-lactamase-producing strains. Moreover, specific infection control practices and regular monitoring of antimicrobial properties of microorganisms, need to prevent the spread and outbreaks of ESBL and MBL-producing bacteria.

5. Conclusion

The incidence of infections due to ESBL and MBL gene-producing bacteria is becoming more and more common in hospitals and the community. Rapid recognition and characterization of different types of resistance might minimize the spread of bacterial infections, and can help select the right choice of medication. Among all the clinical isolates of *Acinetobacter* spp., plasmids may have an additional mechanism for resistance, but there is still a need for further investigation to confirm these results. The current study might be helpful in the treatment and prevention of disease by seeking to improve the efficiency of antibiotics.

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The authors declare no conflict of interest in conducting this study.

References

Aksoy M D, Çavuşlu Ş and Tuğrul H M. 2015. Investigation of metallo beta lactamases and oxacilinases in carbapenem resistant *Acinetobacter baumannii* strains isolated from inpatients. *Balkan Med J*, **32**: 79.

Al-Agamy M H, Khalaf N G, Tawfick M M, Shibl A M and El Kholy, A. 2014. Molecular characterization of carbapenem-insensitive *Acinetobacter baumannii* in Egypt. *Int. J. Infect. Dis.*, **22**: 49-54.

Al-Marjani M, Al-Ammar M and Kadhem, E. 2013. Occurrence of ESBL and MBL genes in Pseudomonas aeruginosa and *Acinetobacter baumannii* isolated from Baghdad, Iraq. *IJCR*, **5**: 2482-2486.

Al-Tawfiq J A and Anani A A. 2009. Antimicrobial susceptibility pattern of bacterial pathogens causing urinary tract infections in a Saudi Arabian hospital. *Chemother.*, **55**: 127-131.

Al Atrouni A, Joly-Guillou M L, Hamze M and Kempf M. 2016. Reservoirs of Non-baumannii *Acinetobacter* Species. *Front Microbiol*, **7**: 49.

Ali N E, Morsi S S and Elgohary E. 2014. Association between antibiotics and disinfectants resistance profiles among *Acinetobacter baumanni* isolates in Zagazig University Hospital's intensive care unit. *Life Sci. J*, **11**: 1-8.

Aqel A A, Meunier D, Alzoubi H M, Masalha I M and Woodford N. 2014. Detection of CTX-M-type extended-spectrum beta-lactamases among Jordanian clinical isolates of *Enterobacteriaceae. Scand. J. Infect. Dis.*, **46**: 155-157.

Beige F, Salehi M B, Bahador N and Mobasherzadeh S. 2015. Plasmid mediated antibiotic resistance in isolated bacteria from burned patients. *Jundishapur J Microbio*, **8**: e13567.

Botts R T, Apffel B A, Walters C J, Davidson K E, Echols R S, Geiger M R, Guzman V L, Haase V S, Montana M A, La Chat C A, Mielke J A, Mullen K L, Virtue C C, Brown C J, Top E M and Cummings D E. 2017. Characterization of four multidrug resistance plasmids captured from the sediments of an urban coastal wetland. *Front Microbiol*, **8**: 1922.

Bush K. 2010. Bench-to-bedside review: the role of β-lactamases in antibiotic-resistant Gram-negative infections. *Crit. Care*, **14**: 224.

Cameranesi M M, Morán-Barrio J, Limansky A S, Repizo G D and Viale A. M. 2018. Site-Specific recombination at XerC/D sites mediates the formation and resolution of plasmid co-integrates carrying a bla(OXA-58)- and TnaphA6-resistance module in *Acinetobacter baumannii*. *Front Microbiol*, **9**: 66.

Cheaito K and Matar G M. 2014. The Mediterranean Region: A Reservoir for CTX-M-ESBL-producing Enterobacteriacae. *Jordan J Biol Sci.*, **7**:1-6

Chung D R, Song J-H, Kim S H, Thamlikitkul V, Huang S-G, Wang H, So T M-K, Yasin R M, Hsueh P-R and Carlos C C. 2011. High prevalence of multidrug-resistant nonfermenters in hospital-acquired pneumonia in Asia. *Am J Respir Crit Care Med.*, **184**: 1409-1417.

Chusri S, Chongsuvivatwong V, Rivera J I, Silpapojakul K, Singkhamanan K, McNeil E and Doi, Y. 2014. Clinical outcomes of hospital-acquired infection with *Acinetobacter* nosocomialis and *Acinetobacter pittii*. *Antimicrob. Agents Chemother.*, **58**: 4172-4179.

CLSI.2017. M100-S27 performance standards for antimicrobial susceptibility testing, twenty-seventh informational supplement. *Clinical and Laboratory Standards Institute, Wayne, PA.*

Delgado-Valverde M, Sojo-Dorado J, Pascual Á and Rodríguez-Baño J. 2013. Clinical management of infections caused by multidrug-resistant *Enterobacteriaceae*. *Ther Adv Infect Dis*, **1**: 49-69.

Dhillon R H and Clark J. 2012. ESBLs: a clear and present danger? *Crit. Care Res. Pract.*, **2012**: 625170.

Eftekhar F, Altayar F and Khidaei H. 2018. Plasmid-mediated class 1 and 2 integron carriage in drug-resistant nosocomial isolates of *Acinetobacter baumannii*. *Arch Clin Infect Dis*, **13**: e57813.

Goyal A, Mani N K, Chahar R, Soni A and Goyal S. 2018. Comparison of various phenotypic methods in detection of carbapenemases and metallo-beta-lactamases in carbapenem resistant clinical isolates of *Acinetobacter* species at a tertiary care centre in North India. *Int. J. Curr. Microbiol. App. Sci*, **7**: 3023-3030.

Hafiz S A F A, Gaikwad A A and Iravane J. 2018. Antimicrobial resistance pattern of pseudomonas aeruginosa of various clinical samples in government medical college, aurangabad, India. *Int. J. Curr. Microbiol. App. Sci*, **7**: 1662-1667.

Hashemizadeh Z, Bazargani A, Emami A and Rahimi M. 2010. *Acinetobacter* antibiotic resistance and frequency of ESBL-producing strains in ICU patients of Namazi Hospital (2008-2009). *JQUMS*, **14**: 47-53.

Howard A, O'Donoghue M, Feeney A and Sleator R D. 2012. *Acinetobacter baumannii*: an emerging opportunistic pathogen. *Virulence*, **3**: 243-250.

Ibrahimagic A, Kamberovic F, Uzunovic S, Bedenic B and Idrizovic E. 2017. Molecular characteristics and antibiotic resistance of *Acinetobacter baumannii* beta-lactamase-producing isolates, a predominance of intrinsic bla(OXA-51), and detection of TEM and CTX-M genes. *Turk J Med Sci*, **47**: 715-720.

Irfan S, Turton J, Mehraj J, Siddiqui S, Haider S, Zafar A, Memon B, Afzal O and Hasan R. 2011. Molecular and epidemiological characterisation of clinical isolates of carbapenem-resistant *Acinetobacter baumannii* from public and private sector intensive care units in Karachi, Pakistan. *J Hosp Infect.*, **78**: 143-148.

Koeleman J G, Stoof J, Van Der Bijl M W, Vandenbroucke-Grauls C M and Savelkoul P H. 2001. Identification of epidemic

strains of *Acinetobacter baumannii* by integrase gene PCR. *J Clin Microbiol.,* **39**: 8-13.

Kuo S C, Chang S C, Wang H Y, Lai J F, Chen P C, Shiau Y R, Huang I W, Lauderdale T L and Hospitals T. 2012. Emergence of extensively drug-resistant *Acinetobacter baumannii* complex over 10 years: nationwide data from the Taiwan Surveillance of Antimicrobial Resistance (TSAR) program. *BMC Infect Dis.,* **12**: 200.

Mathers A J, Peirano G and Pitout J D. 2015. The role of epidemic resistance plasmids and international high-risk clones in the spread of multidrug-resistant *Enterobacteriaceae*. *Clin Microbiol Rev.,* **28**: 565-591.

Nazmul MHM, Jamal H, Rashid MA, Karim Al-Jashamy, Saeid Reza Doustjalali, & Sabet, N. S. 2016. Plasmid profile analysis of clinical isolates of *Acinetobacter* species in Malaysia. *Res J Pharm Biol Chem Sci,* **7**: 1798-1802.

Oberoi L, Singh N, Sharma P and Aggarwal A. 2013. ESBL, MBL and Ampc beta Lactamases Producing Superbugs - Havoc in the Intensive Care Units of Punjab India. *J Clin Diagn Res,* **7**: 70-73.

Owlia P, Azimi L, Gholami A, Asghari B and Lari A R. 2012. ESBL-and MBL-mediated resistance in *Acinetobacter baumannii*: a global threat to burn patients. *Infez Med.,* **20**: 182-187.

Palzkill T. 2013. Metallo-beta-lactamase structure and function. *Ann N Y Acad Sci.,* **1277**: 91-104.

Park S Y and Kim J H. 2017. Clinical significance of extended-spectrum β-lactamase-producing bacteria in first pediatric febrile urinary tract infections and differences between age groups. *Childhood Kidney Dis.,* **21**: 128-135.

Paul S, Jhora S T, Dey P P and Begum B A. 2017. Detection of extended spectrum beta-lactamase (ESBL) producing gram negative bacteria from clinical specimens of sir salimullah medical college and mitford hospital. *Bangladesh J Med Microbiol,* **10**: 8-12.

Pino I, Domínguez Y, González R, Bello T, Sepúlveda A, Mella M, Zemelman M and Zemelman Z. 2007. Extended spectrum beta lactamases (ESBL) production in *Acinetobacter baumannii* strains isolated from Chilean hospitals belonging to VIII Region. *Rev. Chilena Infectol.,* **24**: 137-141.

Porse A, Schønning K, Munck C and Sommer M O. 2016. Survival and evolution of a large multidrug resistance plasmid in new clinical bacterial hosts. *Mol Biol Evol.,* **33**: 2860-2873.

Pournajaf A, Rajabnia R, Razavi S, Solgi S, Ardebili A, Yaghoubi S, Khodabandeh M, Yahyapour Y, Emadi B and Irajian, G. 2018. Molecular characterization of carbapenem-resistant *Acinetobacter baumannii* isolated from pediatric burns patients in an Iranian hospital. *Trop J Pharm Res,* **17**: 135-141.

Sadeghifard N, Ranjbar R and Zaeimi J. 2011. Antimicrobial susceptibility, plasmid profiles, and RAPD-PCR typing of *Acinetobacter* bacteria. *Asian Biomed (Res Rev News),* **4**: 901-911.

Salto I P, Tejerizo G T, Wibberg D, Pühler A, Schlüter A and Pistorio M. 2018. Comparative genomic analysis of *Acinetobacter* spp. plasmids originating from clinical settings and environmental habitats. *Sci. Rep.,* **8**: 7783.

Saranathan R, Pagal Sudhakar R, Singh S K, Shashikala P, Kanungo R and Prashanth K. 2014. Multiple drug resistant carbapenemases producing *Acinetobacter baumannii* isolates harbours multiple R-plasmids. *Indian J. Med. Res.,* **140**: 262.

Selim S A and Hagag N. I. 2013. Analysis of plasmids and restriction fragment length polymorphisms of *Acinetobacter baumannii* isolated from hospitals-AL Jouf Region-KSA. *Inter J Med, Health, Biomed, Bioeng Pharma Eng.,* **7**: 225-231.

Shaikh S, Fatima J, Shakil S, Rizvi S M and Kamal M A. 2015. Antibiotic resistance and extended spectrum beta-lactamases: Types, epidemiology and treatment. *Saudi J Biol Sci.,* **22**: 90-101.

Shoja S, Moosavian M, Rostami S, Farahani A, Peymani A, Ahmadi K and Ebrahimifard N. 2017. Dissemination of carbapenem-resistant *Acinetobacter baumannii* in patients with burn injuries. *J Chin Med Assoc.,* **80**: 245-252.

Sohail M, Rashid A, Aslam B, Waseem M, Shahid M, Akram M, Khurshid M and Rasool M H. 2016. Antimicrobial susceptibility of *Acinetobacter* clinical isolates and emerging antibiogram trends for nosocomial infection management. *Rev Soc Bras Med Trop.,* **49**: 300-304.

Soudeiha M, Sokhn E S, Daoud Z and Sarkis D. 2018. Molecular epidemiology and clonality of *Acinetobacter* spp in a Lebanese hospital over a period of one year. *J Infect Dev Ctries,* **12**: 17S.

Spellberg B and Rex J H. 2013. The value of single-pathogen antibacterial agents. *Nat. Rev. Drug Discov.,* **12**: 963.

Thomson G K, Ayaz M, Lutes K and Thomson K S. 2018. An improved extended-spectrum-beta-lactamase detection test utilizing aztreonam plus clavulanate. *J Clin Microbiol.,* **56**: e01309-17.

Towner K J. 2009. Acinetobacter: an old friend, but a new enemy. *J Hosp Infect.,* **73**: 355-363.

Vahaboglu H, Coskunkan F, Tansel O, Ozturk R, Sahin N, Koksal I, Kocazeybek B, Tatman-Otkun M, Leblebicioglu H, Ozinel M A, Akalin H, Kocagoz S and Korten V. 2001. Clinical importance of extended-spectrum beta-lactamase (PER-1-type)-producing *Acinetobacter* spp. and *Pseudomonas aeruginosa* strains. *J Med Microbiol.,* **50**: 642-645.

Walther B, Klein K-S, Barton A-K, Semmler T, Huber C, Wolf S A, Tedin K, Merle R, Mitrach F and Guenther S. 2018. Extended-spectrum beta-lactamase (ESBL)-producing *Escherichia coli* and *Acinetobacter baumannii* among horses entering a veterinary teaching hospital: The contemporary" Trojan Horse". *PLoS One,* **13**: e0191873.

Weber B S, Ly P M, Irwin J N, Pukatzki S and Feldman M F. 2015. A multidrug resistance plasmid contains the molecular switch for type VI secretion in *Acinetobacter baumannii*. *Proce Nat Acad Sci.,* **112**: 9442-9447.

Wibberg D, Salto I P, Eikmeyer F G, Maus I, Winkler A, Nordmann P, Puhler A, Poirel L and Schluter, A. 2018. Complete genome sequencing of *Acinetobacter baumannii* strain k50 discloses the large conjugative plasmid pk50a encoding carbapenemase oxa-23 and extended-spectrum beta-lactamase GES-11. *Antimicrob Agents Chemother.,* **62**: e00212-18.

Wong D, Nielsen T B, Bonomo R A, Pantapalangkoor P, Luna B and Spellberg B. 2017. Clinical and pathophysiological overview of *Acinetobacter* infections: a century of challenges. *Clin Microbio Rev.,* **30**: 409-447.

Yong D, Shin J H, Kim S, Lim Y, Yum J H, Lee K, Chong Y and Bauernfeind A. 2003. High prevalence of PER-1 extended-spectrum beta-lactamase-producing *Acinetobacter* spp. in Korea. *Antimicrob Agents Chemother.,* **47**: 1749-1751.

Zhang W J, Lu Z, Schwarz S, Zhang R M, Wang X M, Si W, Yu S, Chen L and Liu S. 2013. Complete sequence of the bla(NDM-1)-carrying plasmid pNDM-AB from *Acinetobacter baumannii* of food animal origin. *J Antimicrob Chemother.,* **68**: 1681-1682.

Zurawski D V, Thompson M G, McQueary C N, Matalka M N, Sahl J W, Craft D W and Rasko D A. 2012. Genome sequences of four divergent multidrug-resistant *Acinetobacter baumannii* strains isolated from patients with sepsis or osteomyelitis. *J Bacteriol.,* **194**: 1619-1620.

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Molecular Analysis and Phylogenetic Assessment of the Red Sea Fish of *Plectropomus pessuliferus*

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Abstract

A fish sample related to *Plectropomus pessuliferus* was obtained from Mastoora on the Red Sea in Saudi Arabia, and was identified morphologically by Polymerase Chain Reaction (PCR). The *Plectropomus pessuliferus* sample, locally known as Najil, was 53.8 cm in length and 2110 gm in weight. The sequence alignment of the TMO-4C4 gene (466 bp in length) obtained by PCR revealed a 84 % identity with eleven accessions in GenBank from five different *Plectropomus* species. Analysis of the TMO-4C4 gene sequence alignment showed positional differences of sixty-nine nucleotides with base-pair substitutions and forty-four transversions interchanges, thirteen transitions from A to G, and seven from T to C. Blast protein alignment revealed a 66 % identity between the amino acids' sequence of the TMO-4C4 gene of *Plectropomus pessuliferus* and other five *Plectropomus* species. A nine-amino-acid domain only has appeared in the sample sequence of the current study compared to other *Plectropomus* sequences which may be referred to as a unique sample with new traits. These results should be focused and completed in order to have a good understanding of the genetic information for this fish species.

Keywords*: Plectropomus pessuliferus*, TMO-4C4 gene, base-pair substitutions, Mastoora**,** Red Sea, Saudi Arabia

1. Introduction

Plectropomus pessuliferus (Coral Reef Guide), locally known as Najil, is one of important fish species of the Red Sea which can be found in some countries such as Egypt, Saudi Arabia, Jordan and Sudan; rare species can be found in the Indo-Pacific regions (Ashworth *et al*. 2006). *Plectropomus pessuliferus* fishes have been found to live in coral reef and seaward reefs at a depth range of 25 - 147 m. The *Plectropomus pessuliferus* fish can reach up to a maximum length of 120 cm in the Red Sea and to a minimum length of 63 cm in the Indo-Pacific (Heemstra *et al*. 1993; Morris *et al*. 2000). These large fishes have very variable colors ranging from white or beige to red and the body is covered with blue dots (Durville *et al*. 2003; Randall *et al*. 2003; Sattar *et al*. 2005). This species is rather similar to and is often misidentified as *Plectropomus maculatus*.

The intense fishing pressure on coral-reef fish resources throughout the Red Sea and Indo-Pacific region especially *Plectropomus pessuliferus* (Sluka 2002) creates an urgent need for protection actions in some countries. In the Ras Mohammed National Park (Egypt) *Plectropomus pessuliferus* is protected, as well as in Dungonab and Sanganeb Marine Parks (Sudan). Also, the Ministry of Agriculture of Saudi Arabia has taken measures to protect *Plectropomus pessuliferus* and *Plectropomus areolatus* along the coast, such as prohibiting fishing during the seasons in 1994, 1995, 1999, 2001. Therefore, the landed catch was markedly decreased during these periods (Fallatah 2005).

Fish identification is based not only on morphological features but also on DNA techniques. Santos *et al.* (2013) used mitochondrial and nuclear markers to identify and differentiate between two species of a grouper (*Plectropomus maculatus* and *Plectropomus leopardus*. Harrison *et al*. 2014 used microsatellites to discriminate between two closely-related species of coral reef fish, *Plectropomus leopardus* and *P. maculatus* (Serranidae). The TMO-4C4 gene was previously analyzed in a new *Plectropomus areolatus* fish sample obtained from Yanbu coast on the Red Sea in Saudi Arabia (Gharbawi. 2015).

The aim of this work is to determine and analyze the TMO-4C4 gene sequence in a new *Plectropomus pessuliferus* fish sample obtained from Mastoora on the Red Sea in Saudi Arabia.

2. Materials and Methods

Plectropomus pessuliferus (Red Sea reef fishes) fish sample was obtained from Mastoora on the Red Sea in Saudi Arabia (natural habitat).

2.1. DNA Extraction

A tissue sample of about 2-4 mm^3 was used to extract DNA according to the procedure described in a Puregene DNA isolation kit (Gentra Systems, Minneapolis, MN) (Streelman and Karl 1997).

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2.2. Primer Design and PCR Amplification of the TMO-4C4 Gene

Two specific primers forward (5′ CGCTAATGTTTTACGCTGAG 3′) and reverse (5′ GATGTGTTACCGAGTATTTA 3′) were designed for the TMO-4C4 gene obtained from accession EF517751 of NCBI GenBank. The PCR-amplification reaction was used in a final volume of 25 µL containing 12.5 µL of My *Taq* Red Mix Master mix (BIOLINE), 1.5 µL of 20 mM of each forward and reverse primers, 50 ng of template DNA. PCR conditions started with the initial denaturation step at 94°C for two minutes, followed by forty cycles of thirty seconds at 94°C, the annealing step at 49°C for one minutes, and the elongation step at 72°C for one minute with a final extension of five minutes at 72°C. The PCR amplified product was analyzed using 1.2 % agarose gel electrophoresis in a 1X TBE buffer by staining using 0.8 µg/µL of ethidium bromide, and was visualized under UV light. A TMO-4C4 fragment size of 466 bp was estimated based on a 50 bp DNA ladder (Bioron, Germany).

2.3. Purification and Sequence Alignment of the TMO-4C4 Gene

A 466 bp of the PCR product was purified with the Zymoclean^{Tm} Gel DNA Recovery Kit (Epigenetics Company) according to the manufacturer's instructions. The TMO-4C4 fragment was sent for sequencing, and sequence was compared with the sequences of the most closely-related *Plectropomus* fish samples deposited in GenBank and the sequencing-genome databases using BLAST search (http://www.ncbi.nlm.nih.gov/blast). Highly-conserved residues have a black background, whereas partially conserved residues are shown with a grey shaded background. Numbering at the end of each line refers to the position in the alignment.

2.4. Phylogenetic Analysis

The Kimura's two-parameter model was used to obtain genetic distances (Kimura 1980). The construction of the phylogenetic tree and the dendrogram were obtained using multiple alignment of the TMO-4C4 sequence from *Plectropomus* by the neighbor-joining method (Saitou and Nei 1987) with the Geneious Pro 4.5.4 program.

3. Results

3.1. Morphological Features of the Plectropomus pessuliferus Fish Sample

The *Plectropomus pessuliferus* sample, locally known as Najil, was collected from Mastoora. It is related to the family Serranidae, and was 53.8 cm in length and 2110 gm in weight. This fish sample was orange, and the whole body is covered with blue dots as shown in Figure 1.

Figure 1. *Plectropomus pessuliferus* fish sample obtained from Mastoora on the Red Sea, Saudi Arabia.

3.2. PCR Amplification and Sequence Analysis of the Plectropomus pessuliferus' TMO-4C4 Gene

A fragment corresponding to the partial sequence of the TMO-4C4 gene of an expected size of 466 bp was amplified by PCR from *Plectropomus pessuliferus*. The gene sequence was aligned and compared to GenBank databases using BLAST website. Blast alignment data revealed a 84 % identity between the TMO-4C4 gene sequence of *Plectropomus pessuliferus* and eleven accessions from five different *Plectropomus* species, namely *laevis*, *oligacanthus*, *leopardus*, *maculates* and *areolatus* (Table 1).

Sixty nine base-pair substitutions in nucleotide sequences have been revealed from the nucleotide sequence analysis of the TMO-4C4 gene alignment data compared with other *Plectropomus* species. Forty-four transversions interchanges from purine to pyrimidine and from pyrimidine to purine were detected, while thirteen transitions from purine to purine (A) ⇆ (G), and seven from pyrimidin to pyrimidine (T) ⇆ (C). These results and base-pair substitutions are shown in green color as presented in Table 2 and Figure 2.

Table 1. Blast alignment results for the TMO-4C4 gene sequence of *Plectropomus pessuliferus*

No.	Accession	Description	Identity %
1	KM078024	*Plectropomus leopardus* TMO-4C4 protein (TMO-4C4) gene	84
2	AY949211	*Plectropomus leopardus* TMO4C4 (TMO4C4) gene	84
3	EF517751	*Plectropomus maculatus* TMO-4C4 gene	84
4	EF517747	*Plectropomus leopardus* TMO-4C4 gene	84
5	EF517750	*Plectropomus areolatus* TMO-4C4 gene	84
6	AY949211	*Plectropomus leopardus* TMO4C4 (TMO4C4) gene	84
7	KM078023	*Plectropomus laevis* TMO-4C4 protein (TMO-4C4) gene	84
8	AY949320	*Plectropomus laevis* TMO4C4-like (TMO4C4) gene	84
9	AY949300	*Plectropomus oligocanthus* TMO4C4-like (TMO4C4) gene	84
10	AY949267	*Plectropomus areolatus* TMO4C4 (TMO4C4) gene	84
11	EF517748	*Plectropomus oligacanthus* TMO-4C4 gene	84

Table 2. Nucleotide sequence analysis of the TMO-4C4 gene of *Plectropomus pessuliferus* compared to other *Plectropomus* species.

present in sample as: present in NCBI as:	Nucleotide sequence range	A T	A G	A C	A ---	T A	T C	T G	T ---	C A	C T	C G	G A	G T	G C	G ---
Nucleotide positions change	T=69	29 33 51 53 54 86 100	30 172 249 290 352 263 79	44 74 102 280 396	78 90	19 22 185 201 243 294 337 390	47 81 114 326 372	298 383	165	135 162 191 280 351 357 384	92 186	76 307 334 338	43 138 230 250 283 388	18 48 161 199 228 366 371	225 246 319 380	85 164
		7	7	5	2	8	5	2	1	7	2	4	6	7	4	2

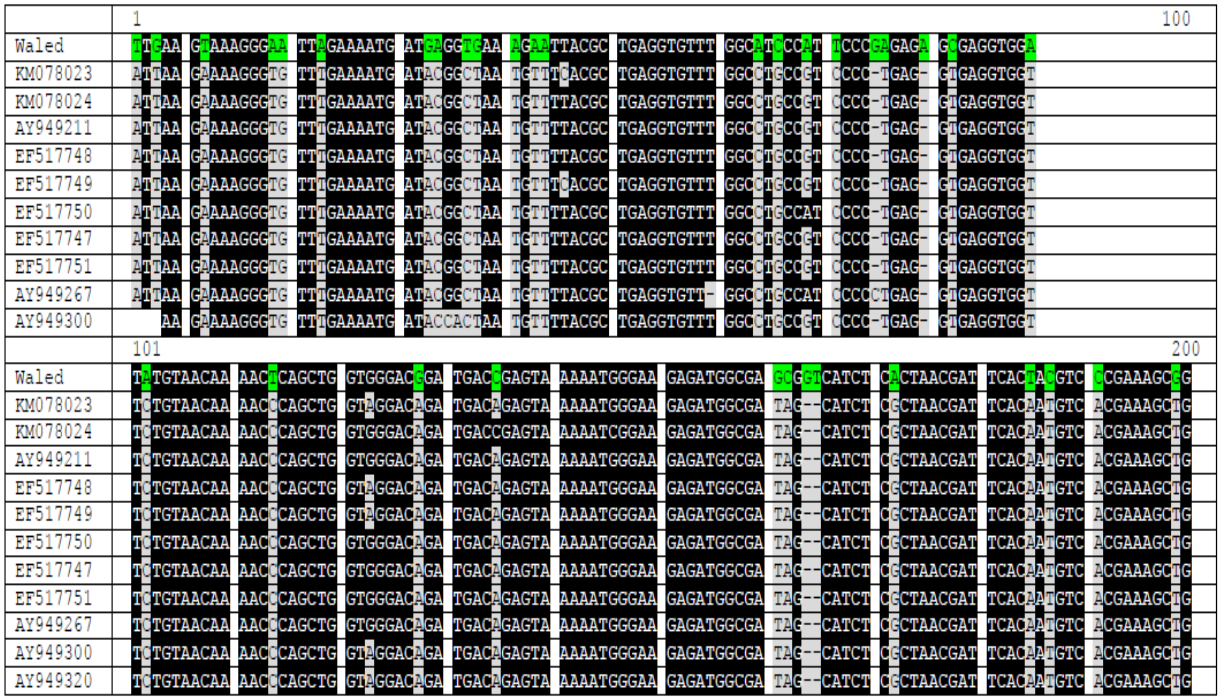

Figure 2. TMO-4C4 gene nucleotides sequence alignment of *Plectropomus pessuliferus* sample of Mastoora compared to other *Plectropomus* species by Blast. Conserved nucleotides are appeared in black, Putative conserved between the different isolates with no identity with isolates are boxed in grey, nucleotides appeared only in our isolate are boxed in green.

3.3. Phylogenetic Tree Based on TMO-4C4 gene Sequence of Plectropomus pessuliferus

The *Plectropomus pessuliferus* TMO-4C4 gene sequence alignment with other *Plectropomus* species obtained from GenBank was used to build a phylogenetic tree in which *Plectropomus pessuliferus* appeared as the main root origin of all other *Plectropomus* species' clusters as shown in Figure 3.

3.4. Analysis of the TMO-4C4 gene Amino Acid Sequence

The TMO-4C4 gene sequence was translated and aligned on GenBank with Blast databases. Six accessions for three *Plectropomus* species, namely *leopardus*, *laevis*, and *areolatus* all revealed a 66 % identity with amino acid sequences of the gene (TMO-4C4) of *Plectropomus pessuliferus* under investigation (Table 3). Amino acid sequences of the TMO-4C4 gene of *Plectropomus pessuliferus* have shown a gap in the first twenty-five amino acids compared to other amino acid sequences of the TMO-4C4 gene of other *Plectropomus* accessions as shown in Figure 4, whereas a nine-amino-acid domain <<< LQKLTPRVf >>> appeared only in the sample sequence of this study compared to the other *Plectropomus* accessions.

Table 3. Identity % of TMO-4C4 translated amino acids sequence from *Plectropomus pessuliferus* compared with other *Plectropomus* accessions.

Accession	Putative protein TMO4C4	Identity %
AAY68548	*Plectropomus areolatus*	66
ABS72105	*Plectropomus leopardus*	66
ABS72107	*Plectropomus laevis*	66
AIJ03033	*Plectropomus leopardus*	66
AAY68519	*Plectropomus leopardus*	66
ABS72105	*Plectropomus leopardus*	66

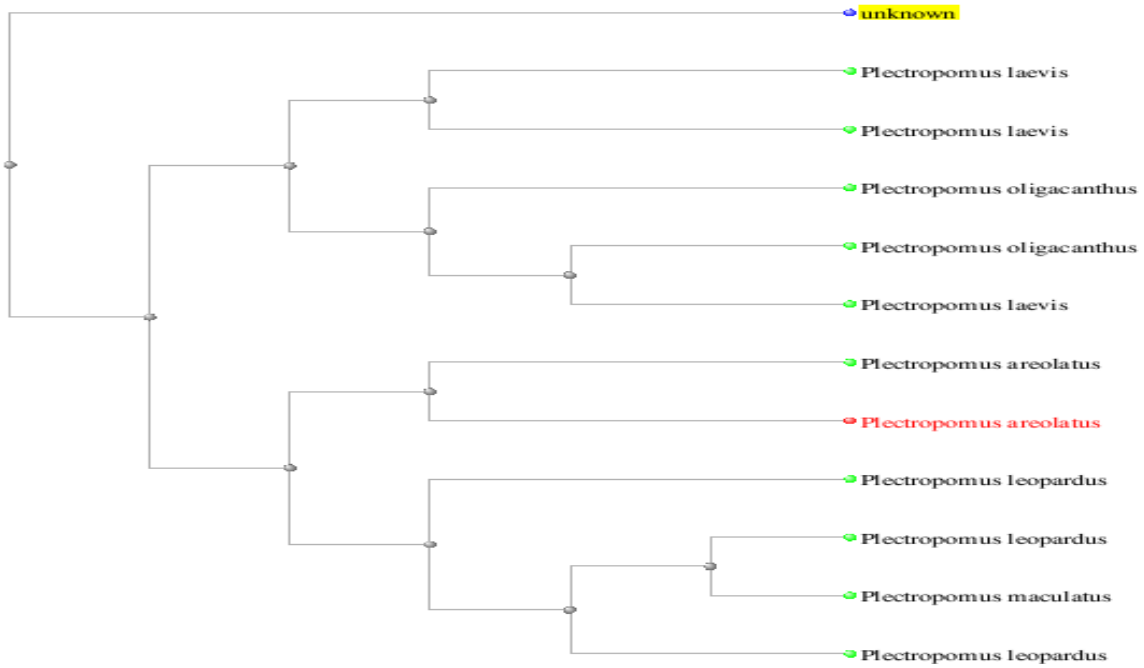

Figure 3. Phylogenetic tree based on alignment of TMO-4C4 gene sequence of *Plectropomus pessuliferus* with other *Plectropomus* accessions.

Waleed	----------SV--FDVLQKLTPRVIEPAPGLRIEVELDENEVKEFEKQVKIITIPEY-------
AIJ03032	RSVALVVVVSQEVRFMPAPPAVTHQHVMEFDVEEDDSSRS-PSPQEILLEVELDENEVKEFEKQVKIITIPEYTADNKSM
AAY68519	RSVALVVVVSQEVRFMPAPPAVTHQHVMEFDVEEDDSSRS-PSPQEILLEVELDENEVKEFEKQVKIITIPEYTADNKSM
AAY68548	RSVALVVVVSQEVRFMPAPPAVTHQHVMEFDVEEDDSSRS-PSPQEILLEVELDENEVKEFEKQVKIITIPEYTADNKSM
AIJ03033	RSVALVVVVSQEVRFMPAPPAVTHQHVMEFDVEEDDSSRS-PSPQEILLEVELDENEVKEFEKQVKIITIPEYTADNKSM
ABS72107	RSVALVVVVSQEVRFMPAPPAVTHQHVMEFDVEEDDSSRS-PSPQEILLEVELDENEVKEFEKQVKIITIPEYTADNKSM
ABS72105	RSVALVVVVSQEVRFMPAPPAVTHQHVMEFDVEEDDSSRS-PSPQEILLEVELDENEVKEFEKQVKIITIPEYTADNKSM

Figure 4. Amino acids' sequence of TMO-4C alignment of *Plectropomus pessuliferus* compared with other *Plectropomus* accessions.

4. Discussion

The morphological features of the *Plectropomus pessuliferus* sample under study agreed with those described by Durville *et al*. (2003) who maintain that this species of fish has very variable colors ranging from white or beige to red and the body is covered with blue. This species is rather similar to and is often misidentified as *Plectropomus maculates http://www.fishbase.se/summary/ Plectropomus-pessuliferus.html*.

However, in morphological taxonomy, characters are delimited usually without any explicit criteria for character selection or coding, and the morphological data sets have the potential to be quite arbitrary. For example, morphologists do not generally report their criteria for including or excluding characters, and when criteria are given, they vary considerably among studies (Wiens 2000). While DNA barcoding such as the TMO-4C4 gene provides taxonomic identification for a specimen, the accuracy of such an assignment depends on whether species are monophyletic with respect to the sequence variations of the genes. That is, individuals of a given species are more closely related to all other conspecifics than to any member of other species. The factors responsible for deviations from taxonomic monophyly may be varied and complex (Funk and Omland 2003); one potential cause of the species-level of polyphyly is the occasional mating between distinct species, resulting in hybrid offsprings carrying a mixture of genes from both parent species. In such cases, combinations of morphological and genotypic data are needed for the species assignment of hybrids. Biological mechanisms, water dynamics, or historical events may cause deep genetic structuring of populations in marine species (Barber *et al.* 2000).

The molecular analysis of the TMO-4C4 gene of an expected size of 466 bp revealed a 84 % identity between the TMO-4C4 gene sequence of *Plectropomus pessuliferus* and eleven accessions from five different *Plectropomus* species, namley *laevis*, *oligacanthus*, *leopardus*, *maculates*, and *areolatus*.

Sixty-nine base-pair substitutions in nucleotide sequences have been revealed from the nucleotide sequence analysis of the TMO-4C4 gene alignment data compared with other *Plectropomus* species. The genetic changes observed in the TMO-4C4 gene of *Plectropomus pessuliferus* suggested that the nucleotides' variation may be attributed to a change in the genetic composition as mentioned by (Gharbawi 2015). Amino acid sequences of the TMO-4C4 gene of *Plectropomus pessuliferus* showed a nine-amino-acid domain. These results suggest a unique sample with new traits which should be fully investigated in order to have a good understanding of the genetic information of this fish species to help those interested in this field including fisheries' management and phylogeographic studies (Williams *et al.* 2003). The nature and extent of genetic changes observed in the TMO-4C4 gene of the *Plectropomus pessuliferus* sample obtained from Mastoora on the Red Sea in Saudi Arabia suggested

that fluctuations in nucleotide sequences are underlain by significant changes in the genetic composition and population integrity. The development of robust hypotheses for phylogenetic relationships within and among coral reef fish will have a major impact on the ability to analyze the evolutionary biology of these colorful, diverse, and ecologically important sea animals.

References

Ashworth JS, Bruce OE and Mohammed EH. 2006. Fish assemblages of Red Sea backreef biotopes. *Aqua Conserv*. **16**: 593-609.

Barber PH, Palumbi SR, Erdmann MV and Moosa MK. 2000. Biogeography: a marine Wallace's line? *Nature* **406**: 692-693.

Durville P, Chabanet P and Quod JP. 2003. Visual Census of the Reef Fishes in the Natural Reserve of the Glorieuses Islands (Western Indian Ocean). *J Mari Sci*., **2**: 95-104.

Fallatah AA. 2005. Trends in catches of *Plectropomus pessuliferus* (Fowler) and *Plectropomus areolatus* (Ruppel) in the Red Sea Coast of Saudi Arabia from 1995 to 2002. *Egypt J Aqua Res.*, **31**: 256-285.

Funk DJ and Omland KE. 2003. Species-level paraphyly and polyphyly: frequency, causes, and consequences, with insights from animal mitochondrial DNA. *Ann Rev Ecol Evolution Systematics* **34**: 397-423.

Gharbawi WY. 2015. Variation of TMO-4C4 nucleotide and protein sequences of *Plectropomus areolatus* (Grouper) sample collected from Yanbu coast on the Red Sea in Saudi Arabia. *Mid-East J Sci Res*., **23**: 1436-1443.

Harrison HB, Feldheim KA, Jones GP., Ma K, Mansour H, Perumal S, Williamson DH and Berumen ML. 2014. Validation of microsatellite multiplexes for parentage analysis and species discrimination in two hybridizing species of coral reef fish (*Plectropomus spp*., Serranidae). *Ecol Evol*., **4**: 2046-2057.

Heemstra PC. and Randall JE. 1993. FAO species catalogue. Vol. 16. Groupers of the world (Family Serranidae, Subfamily Epinephelinae). An annotated and illustrated catalogue of the grouper, rockcod, hind, coral grouper and lyretail species known to date. FAO, Rome.

Kimura M. 1980. A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *J Mol Evol*., **16**: 111-120.

Morris AV., Roberts CM. and Hawkins JP. 2000. The threatened status of groupers (Epinephelinae). *Biodiversity and Conservation*, **9**: 919–942.

Randall JE, Williams JT, Smith DG, Kulbicki M, Tham GM, Labrosse P, Kronen M, Clua E and Mann BS. 2003. Checklist of the shore and epipelagic fishes of Tonga. *Res Bullet*., **502**: 1-37.

Saitou N and Nei M. 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol*., **4**: 406-425.

Santos S, Gomes MF, Ferreira AR, Sampaio I and Schneider H. 2013. Molecular phylogeny of the western South Atlantic Sciaenidae based on mitochondrial and nuclear data. *Mol Phylogenet Evol*., **66**: 423-428.

Sattar SA and Adams MS. 2005. Review of the grouper fishery of the Maldives with additional notes on the Faafu Atoll fishery. Marine Research Centre, Male, Maldives.

Sluka RD. 2002. Grouper and Napoleon wrasse ecology in Laamu Atoll, Republic of Maldives: part 3. Fishing effects and management of the live fish-food trade. *Res. Bullet*., **2001**: 481-493.

Streelman TJ. and Karl SA. 1997. Reconstructing labroid evolution with single-copy nuclear DNA. *Proc R Soc Lond*. **B264**: 1011-1020.

Wiens JJ. 2000. **Phylogenetic Analysis of Morphological Data**, Smithsonian Institution Press, Washington, DC, USA.

Williams AJ, Davies BD and Russ GR. 2003. Scales of spatial variation in demography of a large coral-reef fish-an exception to the typical model. *Fishery Bullet.*, **101**: 673-683.

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