Hashemite Kingdom of Jordan

Jordan Journal of Biological Sciences

An International Peer-Reviewed Scientific Journal

Financed by the Scientific Research Support Fund

http://jjbs.hu.edu.jo/

ISSN 1995-6673

المجلة الأردنية للعلوم الحياتية
Jordan Journal of Biological Sciences (JJBS)
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Jordan Journal of Biological Sciences (JJBS) (ISSN: 1995–6673 (Print); 2307-7166 (Online)): An International Peer- Reviewed Open Access Research Journal financed by the Scientific Research Support Fund, Ministry of Higher Education and Scientific Research, Jordan and published quarterly by the Deanship of Research and Graduate Studies, The Hashemite University, Jordan.

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June 2018

Prof. Khaled H. Abu-Elteen
Editor-in-Chief
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JJBS

Volume 11, Number 4,September 2018
ISSN 1995-6673

Jordan Journal of Biological Sciences

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Isolation of the *Azospirillum* Species from the Rhizosphere of the Leguminous *Bauhinia petersiana in* North Eastern Namibia

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Received November 20, 2017; Revised January 11, 2018; Accepted January 17, 2018

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Abstract

Bacteria associated with the plant roots of leguminous plants have the potential to alleviate the burden of using costly and harmful agrochemicals. Discovery of novel and suitable bacteria for enhancing plant growth is amongst the primary challenges involved in realizing the agronomic application of plant beneficial bacteria. A bacterium isolated from the rhizospheric soil of the legume *Bauhinia petersiana* was described based on several physiological and molecular analyses. The gram-negative bacterium designated as LMAB10 is motile, oxidase positive, catalase negative and displayed lipolytic activity. The bacterium's ability to utilize fourteen compounds as sole carbon sources was examined. A 340 kb fragment of the structural *nifH* gene that encodes for the Fe protein polypeptide of the nitrogenase was amplified from the genomic DNA of isolate LMAB10. Identification of the isolate via 16s rDNA via the BLASTn revealed that the isolate is an *Azospirillum* species with a 98 % similarity to *Azospirillum oryzae* JCM 21588^{T}. Phylogenetic analyses with MEGA5 software showed that the isolate shares an ancestor with *Azospirillum oryzae* JCM 21588^{T} and *Azospirillum zeae* N7^{T}, eventually branching off into a separate taxon. The partially-described isolate from the rhizosphere of *B. petersiana* shows promise for use as a crop inoculant.

Keywords: *Azospirillum* species , *Bauhinia petersiana,* Rhizosphere bacteria , Kavango-Namibia

1. Introduction

The association between plants and various microorganisms within the rhizosphere is a well-documented occurrence that is frequently reported as a beneficial and mutualistic relationship (Kiersi and Denison, 2008). Bacteria are able to make use of the abundant substrates and a low oxygen concentration that is available in the area surrounding the plant roots (rhizosphere), whilst the plant benefits from plant growth-promoting substances and fixed nitrogen provided by the bacteria (Madigan *et al.*, 2014). Associations between the plants and the rhizobacteria suggests that a long term and pronounced extent of adaptation has developed between the two types of organisms in evolutionary time (Nehl and Knox, 2006).

The genus *Azospirillum* is metabolically diverse, and belongs to the *α-Proteobacteria* class of bacteria. This group of bacteria are highly competitive and highly adaptable enabling their survival in various environments. *Azospirillum* spp. are free-living nitrogen fixing microaerophilic bacteria that are well-known to be in association with grasses and cereals (Boddey and Döbereiner, 1988; Baldani *et al.*, 1997; Peng *et al.*, 2006) These bacteria have also been isolated from the soil and from the roots of numerous plants (Ghai and Thomas, 1989; Bilal *et al.*, 1990; Eckert *et al.*, 2001 ; Reis *et al.*, 2001; Lin *et al.*, 2012) *Azospirillum* spp. are cited as biofertilizers (Steenhoudt and Vanderleyden, 2000 ;Gholami *et al.*, 2009) because of their ability to promote plant growth (Vessey, 2003), and are able to moderate osmotic stress in plants (Cassan *et al.*, 2009). To date, there are seventeen *Azospirillum* species reported, though only fifteen are presently valid after *A. irakense* and *A. amazonense* were recently reclassified (Young *et al.*, 2015): *A. lipoferum* (Tarrand *et al.*, 1978) , *A. amazonense* (Falk *et al.*, 1985) , *A. brasilense* (Tarrand *et al.*, 1978) , *A. canadense* (Mehnaz *et al.*, 2007a) , *A. doebereinerae* (Eckert *et al.*, 2001) , *A. fermentarium* (Lin *et al.*, 2013), *A. formosense* (Lin *et al.*, 2012), *A. halopraeferens* (Reinhold *et al.*, 1987), *A. humicireducens* (Zhou *et al.*, 2013), *A. irakense* (Khammas *et al.*, 1989) , *A. largimobile* (Ben Dekhil *et al.*, 1997) , *A. melinis* (Peng *et al.*, 2006), *A. oryzae* (Xie and Yokota, 2005) , *A. picis* (Lin *et al.*, 2009), *A. rugosum* (Young *et al.*, 2008) , *A. thiophilum* (Lavrinenko *et al.*, 2010) and *A. zeae* (Mehnaz *et al.*, 2007b).

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Kalahari bauhinia/ White bauhinia is a leguminous dicotyledonous plant that is able to grow in the dry agro-ecologies of Southern Africa including the Kalahari Desert. *B. petersiana* depends on soil nitrogen as it does not have any root nodules which are the biological apparatus used to fix atmospheric nitrogen by some legumes (Bosch, 2006). Plants are incapable of fixing nitrogen for their own use and therefore are dependent on nitrogen fixing microbes in the rhizosphere and soil to supply nitrogen for plant growth (Reddy, 2014). The plant's components have a variety of uses including medicinal (leaves and roots), animal feed (leaves and seeds), food source (pods, seeds, flowers, bark and roots) and as a dye (roots) (Curtis and Mannheimer, 2005; Bosch, 2006; Chelle, 2011). *B. petersiana* colonises disturbed areas, and has been considered as an arid plant with the potential of becoming a crop in semi-arid areas through domestication efforts and the possibility of reversing desertification effects (Curtis and Mannheimer, 2005; SASSCAL Integrated Science Plan, 2013).

In this study, a bacterium from the rhizosphere of *B. petersiana*, a leguminous shrub found in the wooded grasslands and in the Kalahari of Namibia, was isolated and characterized. The aim was to describe the bacterium associated with the rhizosphere of *B. petersiana*, by using physiological and molecular techniques in order to understand how the plant is able to survive in the soils that are low in nitrogen content.

2. Materials and Methods

2.1. Bacterial Isolation and Physiological Characterization

Rhizospheric soil was collected form *B. petersiana* plants in the East and West Kavango regions of North Eastern Namibia. Soils near the roots of the plants were dug with a trowel sterilized with 80 % ethanol and flaming. The samples were sealed in a sterile plastic container, and stored at ambient temperature until processing. Modified rhizobium LMG 201 medium (Atlas, 2005) was used as the enrichment medium, where the medium was supplemented with vitamin solution (Reinhold *et al.*, 1986), and ammonium chloride was used instead of glutamic acid. Rhizospheric soil (1gram) was aseptically transferred into test tubes containing 9ml enrichment broth medium. The test tubes were kept in a 30±2°C incubator for ten days, with occasional shaking. Spread plates were prepared from serial dilutions on yeast extract mannitol agar (YEMA) and incubated at 30±2°C for seven days. Bacterial isolates were subcultured onto fresh YEMA and Congo red agar (Caceres, 1982) media and incubated at 30±2°C for up to five days. Pure cultures were maintained on fresh agar plates for further observations by incubating at 33±2°C for three days. The gram staining procedure was performed. Bacteria were inoculated on S.I.M. medium (Oxoid Ltd; Hampshire, UK) according to the manufacturer's instructions to determine the motility. The oxidase test was performed by placing a loop full of bacterial colony onto Microbact Oxidase strips (Oxoid Ltd; Hampshire, UK). A colour change from white to blue-violet on the inoculated strip indicated a positive reaction, *Staphylococcus aureus* ATCC® 25923™ was used as a positive control. Three-day old isolates grown on agar plates were submerged in a solution of 3 % hydrogen peroxide (SMM Instruments (Pty) Ltd; Gauteng, SA) to test for catalase activity. The formation of air bubbles ascending from the bacterial colonies was indicative of a positive reaction, *Proteus vulgaris* ATCC® 6380™ was used as a positive control. Bacteria were streaked onto tributyrin agar supplemented with Difco lipase reagent (Difco; Becton, Dickinson and Company Sparks, MD), and incubated at 30±2°C for seven days to test for lipolytic activity. A transparent zone around the edges of the bacterial colony was indicative of lipase activity, *B. subtilis* ATCC® 11774™ and *Staphylococcus aureus* ATCC® 25923™ were used as the positive controls. Two-day old bacterial cultures were inoculated into phenol red carbohydrate broth (MacFaddin, 2000) to determine a carbohydrate utilization summary of the isolate. In this study utilization of the following carbohydrates were tested for: D-fructose (Merck KGaA; Darmstadt, Germany), D-glucose (Saarchem; Gauteng, SA), D-(+)-mannose (Sigma-Aldrich Co; St Louis, MO), D-mannitol (Sigma-Aldrich Co; St Louis, MO), D-(+)-melezitose monohydrate (Calbiochem corp; La Jolla, CA), glycerol (B&M Scientific cc; Capetown, SA), lactose monohydrate (Merck KGaA; Darmstadt, Germany), D+maltose monohydrate (Sigma-Aldrich Co; St Louis, MO), myo-inositol (Sigma-Aldrich Co; St Louis, MO), D-(+)-raffinose pentahydrate (Merck KGaA; Darmstadt, Germany), D-sorbitol (Sigma-Aldrich Co; St Louis, MO), sucrose (Sigma-Aldrich Co; St Louis, MO), D-(+)-xylose (Sigma-Aldrich Co; St Louis, MO), D-(+)-trehalose dehydrate (Sigma-Aldrich Co; St Louis, MO).

2.2. Amplification of nifH Gene Fragment

The genomic DNA of bacteria cultured in Luria-Bertani Broth (AMRESCO; Solon, OH) for three days was isolated using Zymo Research Fungal/Bacterial DNA MiniPrep™ according to the manufacturer's instructions. The nitrogenase *nifH* fragment was amplified by nested polymerase chain reaction (PCR) with primers FGPH19 (5′-TACGGCAARGGTGGNATHG-3′) and PolR (5′-ATSGCCATCATYNTCRCCGGA-3′) for the first step and PolF (5′-TGCGAYCCSAARGCBGACTC-3′) and AQER (5′-GACGATGTAGATYTCCTG-3′) for the second step (Sato *et al.*, *2009*). In the first step a 25µl PCR mixture (12.5µl DreamTaq Green PCR Master Mix 2× (Thermo Scientific; South Africa), 2µl Genomic DNA, 0.5µM of each primer and nuclease-free water) was used for the reaction in a PCR cycle with the following steps: initial denaturation at 94°C for four min, thirty cycles at 94°C for one minute, 55 °C for one minute and 72 °C for two minutes, and final extension at 72 °C for five minutes. For the second step a 25µL PCR mixture (12.5µL DreamTaq Green PCR Master Mix 2× (ThermoScientific), 2µl template from the first step, 0.5µM of each primer and nuclease-free water) was used for the reaction in a PCR cycle with the following steps: initial denaturation at 94°C for four minutes, thirty cycles at 94°C for one minute, 56 °C for one minute and 72 °C for two minutes, final extension at 72 °C for five minutes and hold at 4°C. All amplifications were done in a Bio-Rad MyCycler™ thermal cycler. The PCR products were viewed under UV light after being separated by electrophoresis in a 1.2 % agarose gel stained with ethidium bromide (Sigma-Aldrich Co.; St. Louis, MO).

2.3. 16S rRNA Gene Amplification and Phylogenetic Analysis

Amplification of the 16S rRNA gene fragment was carried out with universal primers 27F (5′-GAGTTTGATCM TGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′) using bacterial genomic DNA as a template (Lane, 1991). The 25µL PCR mixture consisted of 12.5µl DreamTaq Green PCR Master Mix 2× (Thermo Scientific; South Africa), 2µL Genomic DNA, 1µM of each primer and 9.5µL Nuclease-free Water. The reaction was carried out according to (Grönemeyer *et al.*, 2012) in a Bio-Rad MyCycler™ thermal cycler. The PCR cycle steps were as follows: initial denaturation at 95°C for four minutes, thirty-five cycles of denaturation at 95°C for one minute, annealing at 50 °C for thirty seconds, extension at 72 °C for one minute, final extension at 72 °C for ten minutes and hold at 4°C. The products were separated by electrophoresis in a 1 % agarose gel and viewed under UV light. Purification and sequencing of the PCR fragments were carried out by Inqaba Biotec (Pretoria, South Africa). The sequence was analysed in the nucleotide databases using the NCBI's Basic Local Alignment Search Tool program to identify the bacteria and submitted to GenBank (KT944355). Related sequences of *Azospirillum* spp. and *Lacibacterium aquitile* strain LTC-2 were obtained from the NCBI's nucleotide database (www.ncbi.nlm.gov/nucleotide) and included in the multiple alignment using the MEGA5 program (Tamura *et al*., 2011). The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987) via MEGA5 with 1000 bootstrap replicates (Felsenstein, 1985). The evolutionary distances were computed using the Jukes-Cantor method (Jukes and Cantor, 1969).

3. Results

3.1. Cultural and Physiological Characteristics of Isolate

Fifteen distinctive colonies of bacteria were initially isolated from the rhizosphere soil of *B. petersiana.* However, only isolate LMAB10 showed the characteristics of a potential *Azospirillum* isolate when grown on Congo red agar (Caceres, 1982). Thus, isolate LMAB10 was selected for further study. The bacterium exhibited moderate growth on both YEMA and Congo red agar. The colonies of the isolate are convex, generally round with wavy margins, raised edges, and are light pink in colour on YEMA. The appearance on Congo red agar is similar with exception to the colour which is crimson/scarlet in this case (Figure 1). The surface of the colonies are smooth to begin with, but after approximately one week at room temperature, the surface of the colonies seemed dry and scorched while maintaining their colour. Results for the remaining tests and carbon source utilization tests are indicated in Table 1.

Table 1 Physiological and carbohydrate utilization characteristics of isolate LMAB10 and three of its closest relatives.

Test	LMAB10	*A. melinis*	*A. oryzae*	*A. zeae*
Gram	-	-	-	-
Motility	+	+	+	+
Oxidase	+	nd	+	+
Catalase	-	nd	+	+
Lipase	+	nd	nd	nd
Fructose	+	+	+	+
Glucose	+	+	+	variable
Glycerol	+	+	nd	+
Lactose	-	+	-	-
Maltose	+	+	-	-
Mannitol	+	+	-	+
Mannose	-	+	nd	-
Melezitose	+	nd	nd	nd
myo-Inositol	+	+	-	-
Raffinose	-	nd	nd	nd
Sorbitol	-	+	-	+
Sucrose	+	+	-	-
Trehalose	+	+	nd	-
Xylose	+	nd	nd	nd

A. oryzae, *A. melinis* and *A. zeae* data sourced from Xie and Yokota (2005), Peng *et al*. (2006), Mehnaz *et al.* (2007b) and Reis *et al.* (2015).

+ positive; − negative; nd = not determined

3.2. Presence of the nifH Gene and Phylogenetic Relationships with 18 Documented Azospirillum Species

The attempt to amplify the *nifH* gene fragment from the genomics DNA of isolate LMAB10 yielded a PCR product of approximately 340kb in size (image not shown). A BLASTn search of the 16S rRNA gene sequence (1 059 kb) revealed that isolate LMAB10 is most similar to *Azospirillum oryzae* JCM 21588^T (98%) (Figure 2). Based on the 16S rRNA gene sequences, the phylogenetic tree (Figure 3) shows that the isolated bacterium is closely related to the strains *A. oryzae* JCM 21588^T and *A. zeae* N7^T. Nevertheless, isolate LMAB10 is attached to a branch on a different taxon than that of its most similar strains.

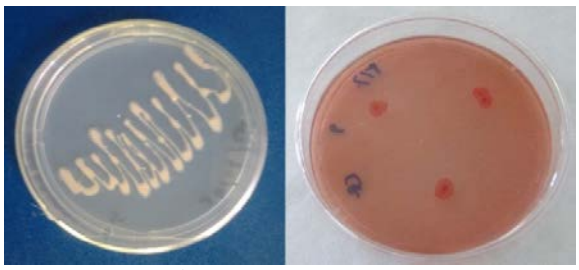

Figure 1. Growth of light pink coloured isolate (LMAB10) on YEMA (left) and crimson coloured spot inoculated colonies on Congo-red agar (right).

Description	Max score	Total score	Query cover	E value	Ident	Accession
Azospirillum oryzae strain JCM 21588 16S ribosomal RNA gene, partial sequence	1820	1820	98%	0.0	98%	NR_117482.1
Azospirillum oryzae strain NBRC 102291 16S ribosomal RNA gene, partial sequence	1816	1816	98%	0.0	98%	NR_114059.1
Azospirillum oryzae strain COC8 16S ribosomal RNA gene, partial sequence	1808	1808	98%	0.0	98%	NR_041233.1
Azospirillum picis strain IMMIB TAR-3 16S ribosomal RNA gene, partial sequence	1770	1770	98%	0.0	97%	NR_042682.1
Azospirillum zeae strain N7 16S ribosomal RNA, partial sequence	1766	1766	98%	0.0	97%	NR_043934.1
Azospirillum melinis strain TMCY 0552 16S ribosomal RNA gene, partial sequence	1759	1759	98%	0.0	97%	NR_043483.1

Figure 2. Highest six BLASTn search results based on the sequence of a 16s rRNA fragment amplified from genomic DNA of isolate LMAB10.

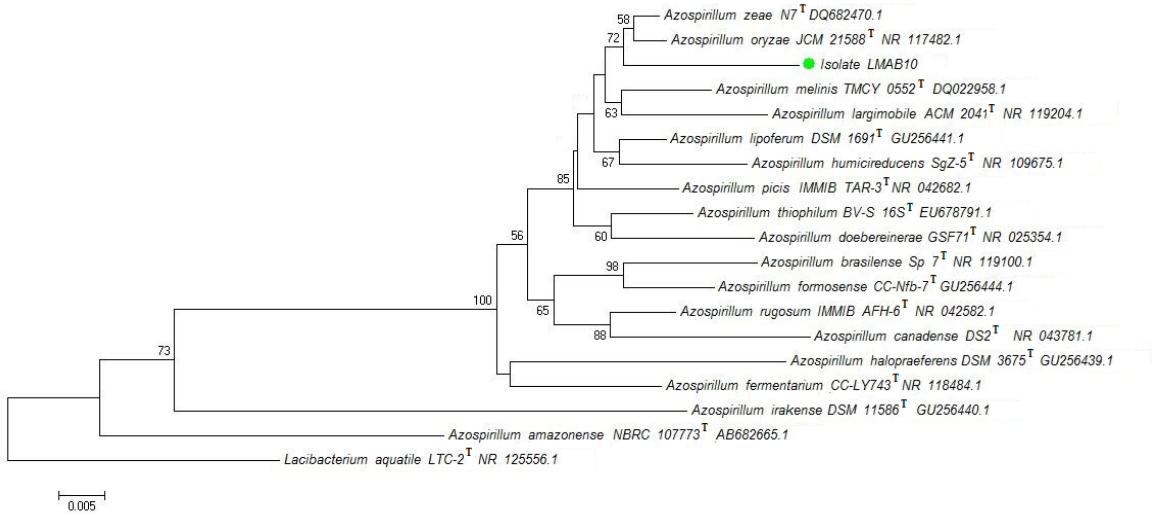

Figure 3. Evolutionary relationship of 17 selected *Azospirillum* strains, isolate LMAB10 and *L. aquatile* strain LTC-2 based on 16S rRNA sequences. *L. aquatile* strain LTC-2 was used as an out-group. The percentage (>50 %) of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. Bar, 0.005 substitutions per nucleotide position were used. The evolutionary history was inferred via the Neighbor-Joining method using MEGA 5.

4. Discussion

Some cultural features belonging to the isolate named LMAB10 are similar to those described by (Young *et al.*, 2008; Lin *et al.*, 2012). A bacterium from the rhizosphere soil of *B. petersiana* was isolated, described and identified as belonging to the *Azospirillum* genus. Results show a 98 % percentage similarity of the 16S rRNA gene to its closest relative *Azospirillum oryzae* JCM 21588^{T}. Though a polyphasic taxonomic approach is necessary to properly distinguish genus members. Based on their polyphasic taxonomic characteristics, *A. irakense* and *A. amazonense* have been described as belonging to taxons outside the *Azospirillum* group and have appropriately been reclassified to *Niveispirillum irakense* and to *Nitrospirillum amazonense* (Prakash *et al.*, 2007; Lin *et al.*, 2014). Closely-related species to isolate LMAB10 are documented to associate with rice (*A. oryzae)*, maize (*A. zeae*) and a molasses grass (*A. melinis*) that grows well in poor nutrient soils of hot environments (Xie and Yokota, 2005; Peng *et al.*, 2006, Mehnaz *et al.*, 2007b). Benefits that the *Azosprillum* spp. confer to cereals include root growth, mitigated osmotic stress, salt stress and drought stress tolerance (Bacilio *et al.*, 2004; Cassan *et al.*, 2009, Bano *et al.*, 2013).

The utilization of numerous carbon sources is a good indicator of a high level of adaptability. The carbohydrate utilization profile also aids in the description of novel species and development of improved culturing and storage media (Reis *et al.*, 2015). As in this case, the differences in the physiological profile between isolate LMAB and its closest relatives support its claim as a novel isolate (Table 1). The isolate LMAB10 was designated as a potential diazotrophic bacterium based on the presence of the amplified *nifH* gene. The *nifHDK* nitrogenase structural genes are one of the most conserved genes within the prokaryotes (Masepohl and Forchhammer, 2007). Most of the *Azospirillum* spp. have demonstrated nitrogen fixation capacity when examined by means of the acetylene reduction assay (Mehnaz, 2015). Microorganism and plants in the nutrient poor arenosol soils of Northern-Eastern Namibia may have more developed relationships than organisms in other soils

(Sato *et al*., 2009, Jones *et al*., 2013). Much like *Tylosema esculentum* (a former member of the genus *Bauhinia*), a semi-arid adapted plant from the Ceasalpiniaceae family, it is highly likely that *B. petersiana* acquires most of its nitrogen from the association with diazotrophic rhizobacteria such as the isolate LMAB10.

Once again the non-specificity of *Azospirillum* spp. association to host plants comes to the fore. *Azospirillum* possess several chemotaxis signal transduction pathways and many receptors more than most soil bacteria, which allows the bacterium to detect an array of signals thus granting it an advantage by making the due adjustments for survival (Hauwaerts *et al*., 2002; Parales and Harwood, 2002; Bible *et al*., 2008; Kaneko *et al*., 2010; Sant'Anna *et al*., 2011; Wisniewski-Dye *et al*., 2012; Alexandre, 2015). The chemotaxis enables the bacterium to locate useful nutrients resources from the plant rhizosphere in order to establish a relationship with the plant in the root zone. The high adaptability, non-pathogenicity to both plants and humans and plant growth promoting properties validate the candidacy of *Azospirillum* spp. for use as biofertilizer constituents (Vacheron *et al*., 2013; Mehnaz, 2015).

5. Conclusion

With a total of 1 % arable land, the promotion of crop agricultural technologies which are sustainably effective and regionally native is vital for the largely semi-arid Namibia. Various plants take advantage of association with *Azospirillum* species in natural settings, therefore, advances can be made to convey and optimize their effectiveness in sustainable agricultural settings. The authors formally conclude that an *Azospirillium* species isolated from the rhizosphere of *B. petersiana* for the first time has been partially described. Consequently, the isolate shows good indications for development into a crop inoculant for an improved plant growth.

Acknowledgments

This study was funded by the Federal Ministry of Education and Research (BMBF) via the Southern African Science Service Centre for Climate Change and Adaptive Land Management (SASSCAL) under Task 051 Improved Soil Fertility Management (Grant number: 01LG1201, Task 051).

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JJBS

Volume 11, Number 4,September 2018
ISSN 1995-6673
Pages 355 - 360

Jordan Journal of Biological Sciences

Phytoremediation Potential of *Chromolaena odorata* (L.) King and Robinson (Asteraceae) and *Sida acuta* Burm. f. (Malvaceae) Grown in lead-Polluted Soils

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Received December 21, 2017; Revised January 18, 2018; Accepted January 23, 2018

Abstract

Heavy metals are non-biodegradable substances that can become deleterious and toxic if accumulated in higher concentrations in the environment. Translocation of nutrients from the plant to the soil is one of the key processes of human exposure to heavy metals through the food chain. The abilities of *Chromolaena odorata* and *Sida acuta* to bioaccumulate and translocate lead (Pb) are studied here under organic fertilizer amendment in order to determine their phytoremediation potentials. The experiment was a factorial combination of Lead at five levels of concentrations in a completely randomized design, replicated three times with two plants and two levels of an organic fertilizer. Seedlings of uniform height were transplanted from the nursery to experimental pots and were grown for ten weeks. The plants were then harvested and dried for the analysis of Pb accumulation both in the soil and the plant tissues using AAS. The results showed that the organic fertilizer enhanced the bioavailability of Pb because all the tested plants displayed a higher absorption of Pb. Significant concentrations of lead were easily taken up by the plants from the soil and were accumulated in the root, while only a small fraction was translocated upwards to the shoots. The two plants exhibited characteristics of a phytostabilizer because their transfer factors were less than one. The uptake of Pb observed in these plants were in the order of: *Sida acuta* > *Chromolaena odorata*, so *Sida acuta* remediates the soil better than *Chromolaena odorata*.

Keywords: AAS, Bioaccumulation, Bioavailability, Plant, Remediate, Soil.

1. Introduction

Heavy metals occur naturally in the ecosystem at varying concentrations due to anthropogenic activities such as metal-smelting, refuse dumping, car exhausts, and the sewage from industries which can all become accumulated to toxic levels (Adekola *et al.,* 2008). The presence of heavy metals in the environment is of great ecological significance due to their toxicity and non-biodegradable nature (Adekola *et al.,* 2008). The heavy metals that are commonly found in ecosystem include: Cadmium, Chromium, Copper, Lead, Mercury, Nickel and Zinc, and exposure to high levels of these metals is known to be hazardous and deleterious to the living organisms in the environment (USEPA, 1997; Hermen, 2011). Lead is the most prominent heavy metal in the soil due to its wide range of applications in human activities (Henry, 2000).

Globally, significant awareness and efforts have been geared towards using specific plants to mop up heavy metals in the ecosystem (Alushllari, 2015). One of the most touted and promising ways of reclaiming soils contaminated with lead is the phytoremediation which involves the use of some plants with significant hyperaccumulating potentials (Baker *et al.,* 2000; Ghosh and Singh, 2005). However, the plants must also be able to produce a large biomass. According to Baker and Brooks (1989), more than 400 plant species belonging to forty-five plant families have been identified and reported from temperate to tropical regions with the ability to tolerate and hyperaccumulate trace elements. One of these plants is *Tithonia diversifolia* whose effectiveness in the remediation of Pb has been reported by Adesodun *et al.* (2010).

Due to the invasive nature and ability of *Chromolaena odorata and Sida acuta* to produce a large biomass, and in addition to the paucity of information on their tolerance to lead toxicity, this study was conducted to unravel the bioaccumulation potentials of these two plants on lead-contaminated soils with organic fertilizer augmentation. This was done with the view to compare the abilities of *Chromolaena odorata* and *Sida acuta* to bioaccumulate and translocate lead. An organic fertilizer was added to the soil to improve its structure and the bioavailability of

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the Pb to the plant for translocation to their aerial parts (Xiao-lang *et al.,* 2011).

2. Materials and Methods

2.1. Study Area

The study was carried out in a screen house in the Biological Gardens of Obafemi Awolowo University campus, Ile – Ife, Osun State, Nigeria. The town Ile – Ife lies within latitude 7^0 30' N – $7^0$35' N and longitude $4^0$30' – $4^0$35' E. The latitude of the study area is 7^0 31' N and the longitude is $4^0$31' E of Ile – Ife, Osun State, Nigeria. The average temperature of the screen house was 35.2^{O}C and the average light intensity was 11380 illuminance.

2.2. Collection of Materials

Samples of the soil were randomly collected from the top 0 – 20 cm depth at the back of Botany Department, Obafemi Awolowo University, Ile-Ife where anthropogenic activities were minimal. The soil samples were air-dried for a week, and thereafter air-dried and sieved using 2 mm mesh gauze to remove debris and stones. Seven – liter plastic pots of 23 cm in diameter were used for the experiment. The plastic pots were then perforated at the base using soldering iron and were filled with 5 kg of soil. Viable seeds of the selected plants *Sida acuta,* and *Chromolaena odorata* were obtained from the field.

2.3. Experimental Design

The experiment was a 3-factorial combination (5×2×2) of five concentrations of Pb, two plants species and two levels (0 and 9.4 g kg^{-1}) of amendment. The Pb concentrations were 0, 200, 400, 800 and 1000 ppm). The amendment was organic fertilizer OBD – Plus. *Sida acuta,* and *Chromolaena odorata* were the plant species used. The experiment was conducted in a completely randomized design with three replicates

2.4. Physical and Chemical Soil Analysis and Seeds Pre-germination

Soil samples were analyzed according to the methods described in Black *et al.* (1965) and Page *et al*. (1985) for some physical and chemical soil properties respectively.

A nursery bed was prepared for the two plant species with viable seeds sown at 2 cm depth. The nursery bed was watered to the field capacity.

2.5. Preparation of Lead Solution

Lead (II) nitrate $\{Pb(NO_3)_2\}$ salt was used as the source of Pb at a concentration of 0, 200, 400, 800 and 1000 ppm. The organic fertilizer OBD-Plus with a Nitrogen (N) content of 0.95 % was used to augment the soil fertility at the concentration of 0 and 9.4 g/kg respectively.

2.6. Pollution of Soil

The bottoms of the plastic pots were perforated to allow aeration and drainage. Plastic trays were placed under each pot for the collection of excess water to prevent nutrient leakages by pouring it back to the experimental pot. After pollution, the pots were left for a week to allow equilibration to set in. Thereafter, an organic fertilizer was applied to augment the soil to improve the soil structure.

2.7. Transplanting the Seedlings to the Polluted Soils

Two weeks after the germination of the seeds in the nursery bed, seedlings with good growth and uniform height were selected and transplanted to each experimental pot at the rate of one seedling per pot.

2.8. Harvesting

At ten weeks after treatment (April, 2014), the plant samples were carefully rinsed under running tap water to remove clogged soil particles. Water droplets were removed from the plant roots using blotting papers. Then, the plant samples were separated into roots and shoots. The soil samples were collected from each experimental pot and prepared for laboratory analyses.

2.9. Analysis of Heavy Metal (Pb)

The soil samples were digested using the Jou (1982) method. The roots and shoots samples were digested using the Audu and Lawal (2005) procedure. Lead concentrations in each of the samples were analyzed spectrophotometrically using Spectronic 20 Absorption spectrophotometer.

2.10. Bioaccumulation and Translocation Factors

The bioaccumulation and transfer factors of the tested plant species were obtained using the equation below:

$$\text{Shoot bioaccumulation factor} = \frac{\text{Concentration of heavy metal in shoot}}{\text{Concentration of heavy metal in soil}}$$

$$\text{Root bioaccumulation factor} = \frac{\text{Concentration of heavy metal in root}}{\text{Concentration of heavy metal in soil}}$$

$$\text{Whole plant accumulation factor} = \frac{\text{Concentration of heavy metal in plant}}{\text{Concentration of heavy metal in soil}}$$

$$Transfer\ factor = \frac{\text{Concentration of heavy metal in shoot}}{\text{Concentration of heavy metal in root}}$$

(Cui *et al.,* 2007 and Yoon *et al.,* 2006).

2.11. Statistical Analysis

Data were subjected to analysis of variance (ANOVA), and means were separated using Fisher's LSD at 5 % probability level. All analyses were carried out using SAS version 9.1.

3. Results

3.1. Physical and Chemical Properties of the Soil Used in the Experiment

The physical and chemical characteristics of the soil used in the screen house are presented in Table 1. The texture of the soil was loamy sand. The pH in 1:1 soil to water ratio was 5.8 for the top soil indicating a slightly acidic soil condition. The organic carbon content of the soil was 40.10 g kg^{-1}, while the nitrogen, phosphorus and potassium values were 3.20 g kg^{-1}, 10.4 mg kg^{-1} and 2605 mg kg^{-1} respectively. Moreover, Calcium (Ca) 31.0 mg/kg, and magnesium (Mg) 2432.5 mg/kg, and Lead (Pb) 0.098ppm were found in the soil samples.

Table 1. Physical and Chemical Characteristics of the soil used in the study

Characteristics	Value
pH (H_2O)	5.8
Organic carbon (g kg^{-1})	4.10
Nitrogen (g kg^{-1})	3.30
Clay (%)	6.8
Silt (%)	4.0
Sand (%)	89
Phosphorus (mg kg^{-1})	10.4
Ca^{2+} (mg kg^{-1})	31.0
Mg^{2+} (mg kg^{-1})	2432.5
K^{+} (mg kg^{-1})	2605
Na^{+}(mg kg^{-1})	272.5
Lead (mg kg^{-1})	0.098

3.2. Bioaccumulation Concentration of Pb in the Tissue of the Plant Species

The lead (Pb) concentration in the plants tissue increased as the lead concentration in the soil increased, as shown in Table 2. The lead concentration in the soil also decreased. The concentration of extractable lead in the soil under all treatments decreased between 39.5 %-55.8 % and 53.6 %-65.9 % in *Sida acuta* without and with the fertilizer application respectively. Also 38.5 %-51.8 % and 46.6 %-62.2 % in *Chromolaena odorata* without and with the fertilizer application respectively. *Sida acuta* extracts more lead from the soil followed by *Chromolaena odorata*.

Table 2. Bioaccumulation of Pb in the tissue of the plant species and the soil after harvest.

Pb concentration (ppm)		Bioaccumulation concentration of Pb (ppm)					
		Sida acuta			*Chromolaena odorata*		
		Shoot	Root	Soil	Shoot	Root	Soil
NF	0	0.0^{e}	0.0^{e}	0.1^{e}	0.0^{e}	0.0^{e}	0.4^{e}
	200	28.8^{d}	54.3^{d}	121.0^{d}	16.7^{d}	41.6^{d}	123.1^{d}
	400	55.7^{c}	96.1^{c}	232.1^{c}	35.2^{c}	77.1^{c}	270.0^{c}
	800	192.3^{b}	275.0^{b}	438.4^{b}	152.8^{b}	237^{b}	451.8^{b}
	1000	255.7^{a}	290.4^{a}	442.1^{a}	181.1^{a}	256.8^{a}	482.3^{a}
	LSD	0.08	0.78	0.78	0.08	0.78	0.77
F1	0	0.0^{e}	0.0^{e}	0.2^{e}	0.0^{e}	0.0^{e}	0.2^{e}
	200	48.6^{d}	82.1^{d}	92.8^{d}	24.6^{d}	61.6^{d}	106.9^{d}
	400	64.3^{c}	106.8^{c}	207.0^{c}	43.2^{c}	83.4^{c}	220.8^{c}
	800	197.1^{b}	310.3^{b}	329.8^{b}	156.1^{b}	271.2^{b}	361.1^{b}
	1000	327.4^{a}	373.2^{a}	341.3^{a}	247.5^{a}	352.1^{a}	378.0^{a}
	LSD	0.08	0.50	0.95	0.08	0.09	0.78

NF = No fertilizer, F= Fertilizer. Means with the same letters within the column are not significantly different at $p < 0.05$

3.3. Bioaccumulation Factors of the Shoot

The shoot bioaccumulation factors (BCF) for the two plant species were presented in figure 1. The BCF for the shoots of the two plants increased with an increase in the contaminant concentrations as shown in figure 1a without the fertilizer application. Highest (0.6) bioaccumulation factor for the shoots was reported in *Sida acuta*, while shoot BCF of *Chromolaena odorata* was 0.4. Under organic fertilizer amendments (figure 1b), the BCF of *Sida acuta* increased by 67 %, while that of *Chromolaena odorata* increased by 17 % across the two plant species, the organic fertilizer amendment improved the shoot BCF.

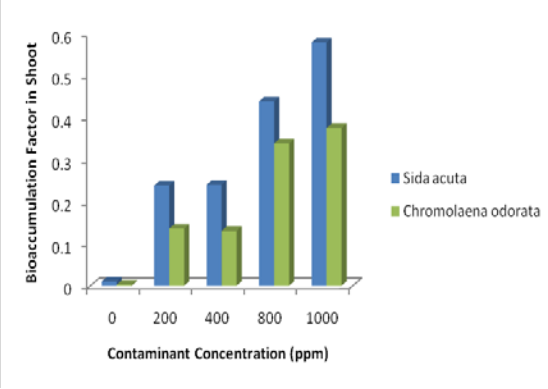

Figure 1a. Bioaccumulation factor for shoots without fertilizer application

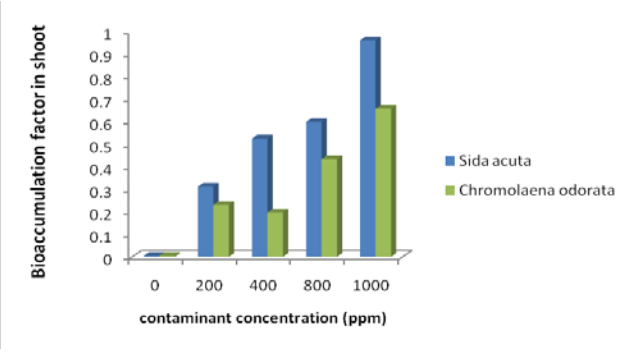

Figure 1b. Bioaccumulation factor for the shoots with the fertilizer application

3.4. Bioaccumulation Factors for the Roots

In figure 2, the BCFs for the roots of the two plant species with and without the organic fertilizer augmentations were presented. The highest was 0.7 in *Sida acuta* and 0.53 in *Chromolaena odorata* without fertilizer application as shown in figure 2a. With the organic fertilizer (figure 2b), the highest bioaccumulation factor for the roots was 1.1 in *Sida acuta* and 0.9 in *Chromolaena odorata*. Plants treated with the organic fertilizer had the highest bioaccumulation factor for the roots. For example, at a low-pollution strength of 200 mg kg^{-1}, the root BCF for *Sida acuta* was 0.52, while that of the *Chromolaena odorota* was almost 50 % less when the two plants were grown at the same pollution strength. Even without the fertilizer application, root BCF of *Sida acuta* was 41 % more than that of the *Chromolaena odorota*. Similarly, at the highest pollution strength of 1000 mg kg^{-1} ,*Sida acuta* accumulated more of the lead both in the roots and shoots than that of *Chromolaena odorota* with root BCF being less than about 42 % increase both with and without the fertilizer application.

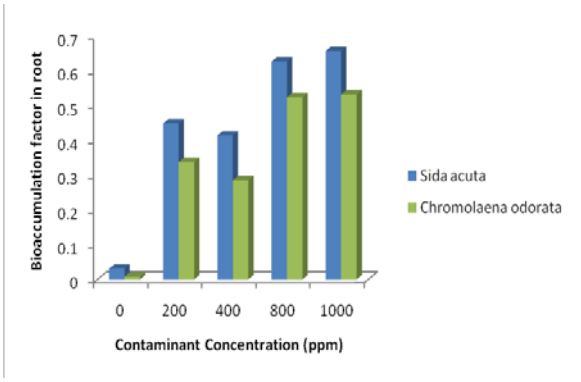

Figure 2a. Bioaccumulation factors for roots without fertilizer application

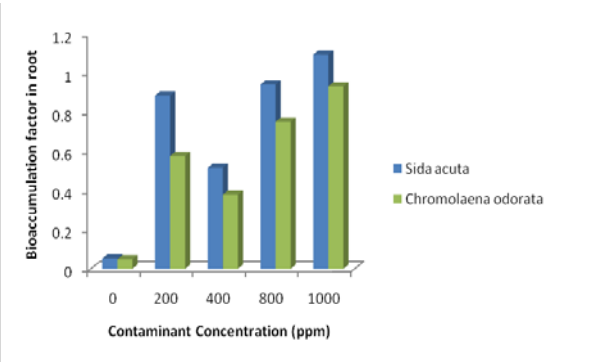

Figure 2b. Bioaccumulation factors for roots with fertilizer application

3.5. Bioaccumulation Factors for the Whole Plants

The bioaccumulation factors for the whole plants increase as the concentration of the contaminant increases, as shown in figure 3. The highest bioaccumulation factors for the whole plants were 1.2 in *Sida acuta* and 0.9 in *Chromolaena odorata* without the fertilizer application (figure 3a). With the organic fertilizer, the highest bioaccumulation factors were 2.1 in *Sida acuta* and 1.6 in *Chromolaena odorata*. Plants grown with the organic fertilizer (figure 3b) have the highest bioaccumulation factor for the whole plants. For *Chromolaena odorata*, the highest BCF value (0.9) was less than one while under fertilizer application. BCF values above one were reported with 31 % less than that obtained for *Sida acuta* exposed to the same pollution stress. BCF for the whole plant showed that the highest BCF was reported in *Sida acuta* grown in 1000 mg kg^{-1} of Pb polluted soils with and without fertilizer application.

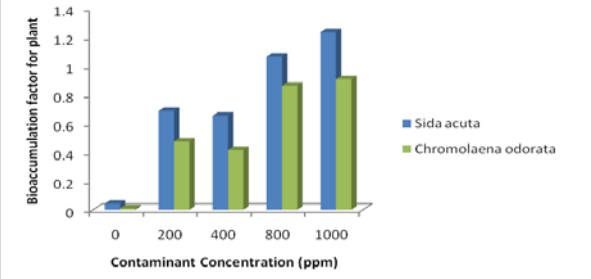

Figure 3a: Bioaccumulation factors for plants without fertilizer application

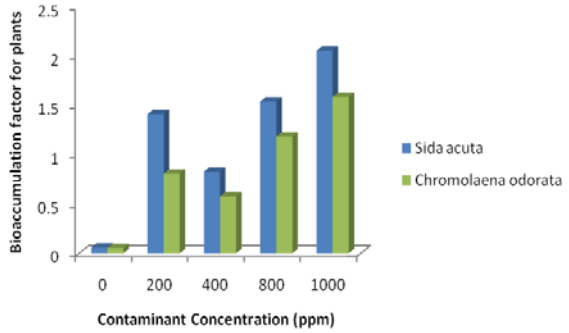

Figure 3b, Bioaccumulation factors for plants with fertilizer application

3.6. Transfer Factor (TF)

The TF of the plants increased with an increase in the contaminant concentration as shown in figure 4. The highest was 0.9 in *Sida acuta* and 0.7 in *Chromolaena odorata* under without fertilizer application (figure 4a). With the organic fertilizer application (figure 4b), the highest was 0.9 in *Sida acuta* and 0.7 in *Chromolaena odorata*. The TF were the same under the fertilizer and without fertilizer application for each plant.

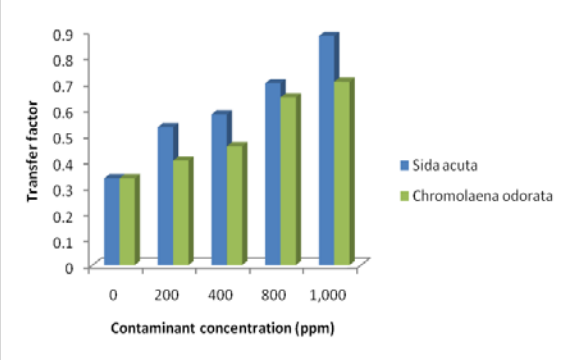

Figure 4a. Transfer factors of the plants without fertilizer application

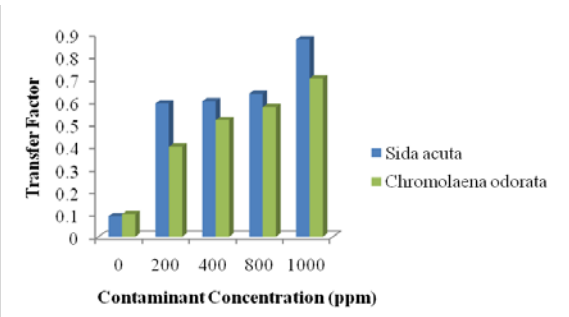

Figure 4b. Transfer factors of the plants with fertilizer application

4. Discussion

The success of the phytoremediation process through the techniques of phytoextraction of the contaminant depends largely on the ability of the candidate plant to transfer contaminants from the below-ground biomass to the aboveground, In contrast, the success of the phytoremediation process through the techniques of phytostabilization of the contaminant depends largely on the restrictive ability of the plant to stabilize the

contaminants below-ground biomass (Baker, 2000; Dada and Awotoye, 2013).

In this study, *Sida acuta* and *Chromolaena odorata* showed varied bioaccumulation potentials after exposure to different concentrations of lead pollution. In addition, the level of the lead contamination determines the Pb-uptake in all the studied plants. This observation was similar to that reported by Wang *et al.* (2007) for *Bidens maximowicziana* and Oseni *et al*. (2015) using *Momordica charantia.* This may be because the roots of plant are the first to have direct contact with the contaminants through passive transport. The current study comes in agreement with previous studies such as Blaylock *et al.* (1997); Salido *et al.* (2003); Aiyesanmi *et al.*(2012) where a higher Pb absorption was observed in the plant species grown in soils treated with an organic fertilizer. This implies that *Chromolaena odorata* and *Sida acuta* were able to absorb and bioaccumulate significant concentrations of lead in this study. Also, it was found that the organic fertilizer enhanced the bioavailability of Pb in the soil-environment.

Considering the BCF of *Sida acuta Chromolaena odorata* under the organic fertilizer application tend to accumulate in the roots more than in the shoots. This was in line with the findings of Parsadoost *et al.* (2008), Cho-Ruk (2006) and also the work of Mojiri (2011) when studying the potentials of Corn *(Zea mays)* in the phytoremediation of soil contaminated with Cadmium and Lead using EDTA for the bioavalability of the heavy metal ions. Moreover, Adejumo *et al.* (2011) in their study of *in-situ* remediation of heavy-metal contaminated soils using the Mexican sunflower (*Tithonia diversifolia*) and cassava waste as composts reported a similar trend in their study. Likewise, the work of Wang *et al.,* (2007) revealed that *Bidens maximowicziana* is a new hyperaccumulator of lead (Nie *et al*., 2004). However, EDTA application had also been reported to enhance the phytoremediation of lead by *Bidens maximowicziana* when planted in lead-contaminated soils. Lead-uptake studies in plants have demonstrated that the roots have an ability to take up significant quantities of Pb while simultaneously restricting its translocation to the aboveground parts (Oseni *et al.*, 2015). The majority of the lead was easily taken up by plants from the soil and accumulated in the roots, while a lesser fraction was translocated upwards to the shoots (Patra *et al.*, 2004). This may be attributed to the fact that passive transport of ions occurred in the roots which were directly exposed to ionic environment. This may extend to the endodermis but not beyond because of the casparian strip. According to Mojiri (2011), the capacity of the soil to adsorb lead increases with increasing of pH, cation exchange capacity (CEC), organic carbon content, soil/water Eh (redox potential), and phosphate levels. Some ways to induce Pb solubility are to decrease the soil pH and lower its organic matter because Pb binds with organic material in the soil (McBride, 1994; Sharma and Dubey, 2005).

The results of the current study revealed BCF of 2.1 and 1.6 for *Sida acuta* and *Chromolaena odorata* respectively. The BCF were higher than one in the two plants, depicting their potentials to accumulate Pb from the soil. These results respond to the work of Pitchtel *et al.* (2000) when analyzing Pb concentrations in plants collected from a dump site. Similarly, Stoltz and Greger (2002) reported a range of 3.4 to 920 mg kg^{-1} of Pb concentrations in different wetland plant species collected from mine tailings. So, plants exhibiting bioaccumulation factors greater than one are suitable for phytostabilization of contaminants. According to Fitz and Wenzel (2002), they are tolerant plants which restrict root–shoot transfers.

Another important mobility index that should be taken into consideration when assessing the phytoremediation potentials of any plant is the ability of the plants to translocate metals from the roots to the shoots which can be measured using a mobility index referred to as the transfer factor (TF). Enrichment occurs when a contaminant taken up by a plant is not degraded rapidly, resulting in an accumulation in the plant (Baker, 2000; Dada and Awotoye, 2013). The highest transfer factors of the studied plants were were less than one, which shows that the plants restricted the Pb transfer from their roots to the shoots. This observation may be attributed to the effects of Pb toxicity. Tang *et al.* (2009) indicated that in *Arabis paniculata*, with the elemental Pb concentration of 9- 267 µM, the transfer factor of the plant was below one. Moreover, Aiyesanmi *et al.* (2012) also reported BCF and TFs of less than one in Lead accumulation in Siam weed (*Chromolaena odorata*), Nodeweed (*Synedrella nodiflora*), and Water leaf (*Talinum triangulare*).

The two plants used in this experiment absorbed a significant concentration Of lead into their root tissues under the fertilizer application with TFs being less than one. This implies that the two plants exhibit characteristics of a phytostabilizer. However, the uptake of Pb in these plants were in the order *Sida acuta* > *Chromolaena* odorata. Thus, the treatment of the soil with the organic fertilizer enhanced the uptake of lead in the plants. This is because the spiking of the soil environment with an organic fertilizer increases the bioavailability of lead in the soil solution.

5. Conclusion

The two plants used in this experiment showed a significantly higher absorption of lead. It could also be observed that the treatment of the soil with the organic fertilizer enhanced the uptake of Pb in the plants by increasing the bioavailability of Pb in the soil solution. The two plants exhibited the characteristics of a phytostabilizer because their transfer factors were less than one. The uptake of Pb observed in these plants were in the order *Sida acuta> Chromolaena odorata* both in the roots and the shoots and this order holds both with organic fertilizer and without organic fertilizer soil.

Acknowledgments

The authors express their appreciation for the efforts of Mr Biodun Omole for the assistance rendered during the research and the staff of Obafemi Awolowo University at Biological gardens who helped in monitoring and watering the plants in the screen house.

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JJBS

Volume 11, Number 4,September 2018
ISSN 1995-6673
Pages 361 - 368

Jordan Journal of Biological Sciences

The Correlation between Plasmid and Metal and Multi-Drug Resistance in Bacteria from Streams Impacted by Husbandry and Wastewaters in Akure, Nigeria

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Received November 30, 2017; Revised January 10, 2018; Accepted January 29, 2018

Abstract

Freshwaters are continuously subjected to natural and anthropogenic influences including heavy metals and antibiotic wastes that do not only pose a high level of multigenetic adaptations in microorganisms, but also forecast life-threatening consequences such as chronic public health risks and ineffective disease managements in human and veterinary medicines. The current study investigated bacterial isolates from surface waters and profiled them for metal, antibiotic resistance and plasmids. Antibiotics' susceptibility test was carried out using the disk diffusion technique and the metal resistance test by incorporating different concentrations of Zn, Fe, Cd and Ni solutions into Mueller-Hinton agar plates. The plasmid extraction and electrophoresis of the DNA were carried out on 0.8 % agarose gel in a 0.5X concentration of Tris-Borate-EDTA (TBE) buffer. To demonstrate the role of plasmid in resistance, the isolates were subjected to plasmid curing using sodium dodecyl sulfate (SDS). The identities of the isolates revealed by the 16S rRNA sequencing analysis are: *Proteus mirabilis*, *Alcaligenes faecalis*, *Alcaligenes faecalis faecalis*, *Bacillus cereus*, *Stenotrophomonas acidaminiphila*, *Proteus penneri*, *Lysinibacillus macrolides* and *Serratia* sp. Results revealed that all the isolates were multidrug-resistant to the different antibiotics tested, and that 62.5 % of the isolates were found to harbor plasmids. Multi-resistance to the antibiotics was conservatively plasmid-borne or chromosomally-mediated. Multi-resistance to the antibiotics was conservatively plasmid-borne or chromosomally-mediated. Multiple broad spectrum antibiotic resistance indices of the isolates ranged from 0.357 to 0.786. Plasmid-mediated heavy metal resistance was recorded in *A. faecalis faecalis, P. mirabilis and B. cereus* to varying metals concentration, while heavy-metal-multi-resistance was chromosomally-mediated in *P. penneri, S. acidaminiphila* and *A. faecalis faecalis* to all the metals. The study forewarns against the health risks of contracting surface water bacteria especially by the users of the water resources and through food chains.

Keywords: Surface water, Bacteria, Multi-drug resistance, Metal resistance, Plasmid.

1. Introduction

The inaccessibility to wholesome sources of water supply has necessitated the use of freshwaters in most regions of the world especially developing countries. In the recent past, the expanding human population, industrialization, intensive agricultural practices and discharges of massive amounts of wastewater into the rivers and streams have resulted in the deterioration of water quality (Nurcihan and Basaran, 2009).

The impact of these anthropogenic activities has been so extensive that water bodies have lost their self-purification capacity to a large extent (Sood *et. al*., 2008).

According to Shoeb (2006), the main sources of heavy-metal pollution in aquatic bodies are usually linked with areas with congested industries and high automobile use. Heavy metals as natural components of the earth's crust are increasingly found in microbial habitats due to several natural and anthropogenic processes. However, microbes have developed mechanisms to tolerate the presence of heavy metals either by efflux, complexation or reduction of metal ions, or through using them as terminal electron acceptors in anaerobic respiration (Mgbemena *et al.*, 2012).

Human and animal wastes serve as major sources of entry of pathogens into surface waters. Entry of pathogens into rivers can occur through rainwater surface run-offs, storm sewer spillages or overflow, discharge of untreated or partially-treated effluents from wastewater treatment plants (Petersen *et al.*, 2005; Donovan *et al.*, 2008). Nigoyi, (2005) stated that the impact of water pollution on human health depends mainly on the water uses, as well as the concentration of pathogens in the water.

Due to the overuse of antibiotics to treat microbial infections, microorganisms are becoming highly resistant to the available antibiotics. The uncontrolled or inappropriate use of antibiotic drugs in both industrialized and developing countries have contributed to the rapid development of resistance in pathogenic microbes.

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Plasmids are extra chromosomal DNA molecules. They are circular DNA molecules that replicate independently of the bacterial chromosome (Wang *et al*., 2011; Adeyemo *et al*., 2015).

The plasmids that carry genes conferring on the host cell resistance to antibiotics are called as 'R factors.' R factors are encountered in certain strains of almost all pathogenic bacteria. These R factors can be transferred to other bacteria in the environment and these extrachromosomal DNA sequences are responsible for the emergence of multiple drug resistant (MDR) strains (Barrow *et al.,* 1986).

Studies have shown the growing concerns regarding water borne diseases as a result of human interaction with surface water. In the report of Bolaji *et al.,* (2011), the greatest threat to the use of antibiotics is the emergence and spread of resistance in pathogenic bacteria that consequently cannot be treated by previously successful regimens. Available therapeutic options for antibiotic resistant organisms are severely limited, as these organisms frequently display a multidrug resistant (MDR) phenotype (Okonko *et al.,* 2009). It is thought that a correlation exists between metal tolerance and antibiotic resistance in bacteria as Mgbemena *et al.,* (2012) and Amalesh *et al.,* (2012) have previously isolated and investigated multidrug resistance and metal resistance in bacteria from municipal waste.

The issue of the emergence of the resistance phenomenon in bacteria justifies research on the multidrug and metal resistance of bacteria to serve as a pathway to the development of new possible anti-infective agents or resistance modifiers, and also give a lead on the ability of some bacteria that have potentials to act as a bioremediating agent in metal- polluted environments.

2. Materials and Methods

2.1. Surface Water Bacterial Isolates

Eight isolates previously isolated from surface waters in an earlier investigation were selected for this study among stocked cultures. The selected surface waters (FUTA and Onyearugbulem streams) have been highly impacted with husbandry and abattoir wastewater effluents due to location of major city abattoirs 10m away from the streams. The isolates have shown varying degrees of resistance to conventional antibiotics (data not shown).

2.2. Resuscitation and Presumptive Identification of Surface Water Isolates

The bacterial isolates were sub-cultured onto a freshly prepared nutrient agar plates, and were incubated at 37^{o}C for twenty-four hours. Pure culture of the isolates was further confirmed before preservation on agar slants at 4^{o}C for further analysis (Cheesebrough, 2006).

2.3. Molecular Characterization of the Isolates

A PCR-based characterization of the isolates was carried out. Genomic DNA of the isolates was extracted and purified according to Gurakan *et al.,* (2008). DNA concentration was then estimated spectrophotometrically by comparing band intensities with the ladder of known concentration on agarose gel. Also, the 16S rRNA gene of the isolates was amplified as described by Sambrook *et al.,* (2001) using the primer pair 27F-5′-AGAGT TTGATCCTGGCTCAG-3′, and 1492R 5′GGTTACC TTGTTACGACTT-3′.

2.4. Sequencing and Analysis of 16SrRNA Gene Report

The 16SrRNA gene amplified products were purified with Exo sap, and were then sent for DNA Sanger sequencing. The sequence data were analysed using the ABI Sequencing Analysis software (version 5.2), and were subjected to the basic local alignment search tool on the NCBI Genbank website (www.ncbi.nlm.nih.gov). Nucleotide sequences generated from each amplified 16S rRNA gene were submitted to the Genbank Nucleotide Sequence Database.

2.5. Plasmid Extraction

Plasmid extraction was carried out using the method described by Ojo and Oso (2009). Pure isolates were inoculated on tars broth and were incubated. The grown cells were harvested and suspended in 200 µL of solution A [100 mm glucose-50 mm tris hydrochloride (pH8)-10mm EDTA containing 10mg tanolysin and were incubated for thirty minutes at 37ºC in a dodecyl sulphate; 0.2 µL NaOH was added, and the samples were mixed by inverting the tubes. Exactly 300µL of a 30 % potassium acetate solution (pH 4.8) was added, and the samples were mixed by vortex. The supernatant was removed, extracted once with a phenol – chloroform mixture (1:1), and precipitated with an equal volume of isopropanol. The plasmid DNA was then dissolved in 100 µL of tris-EDTA buffer. Electrophoresis of the DNA was carried out on a 0.8 % agarose gel in a 0.5×concentration of trisborate-EDTA buffer. After boiling, the solution was allowed to cool; 10 µL of ethidium bromide was added to the cooled agarose solution. This was poured into a casting tray with a comb placed across its rim to form wells. The gel was allowed to set for 30 minutes, and the comb was carefully removed. Exactly 20 µL of the plasmid DNA samples were then loaded into the wells after mixing with 2 µL of bromophenol blue. The gels were thereafter electrophoresed in a horizontal tank at a constant voltage of 60V for about 90 minutes. After electrophoresis, plasmid DNA bands were viewed by the fluorescence of bound ethidium bromide under a short wave ultraviolet light transilluminator, and the photographs were taken using a digital camera.

2.6. Plasmid Curing

The plasmids were cured by treatment with sodium dodecyl sulphate (SDS) according to the method of Al Sa'ady *et al.,* (2014). The nutrient broth was prepared and supplemented with 3.0 % concentration of SDS. An overnight culture of the bacteria cultures was added into the curing agent supplemented broth. The samples were then incubated at 37 °C for 24 hours under constant agitation. The isolates were subcultured onto Mueller Hinton agar, and the plasmid extraction was repeated on the microorganisms to verify if the plasmids were successfully cured.

2.7. Metal and Multidrug Profiling

2.7.1. Determination of Antibiotics Resistance

The disk diffusion technique was employed for antibiogram testing (Willey *et al.* 2008). Standardized

inoculum size (0.5 McFarlands) corresponding to 1.5 x 10^8 CFU/mL of both wild and the plasmid-cured isolates was swabbed onto Mueller Hinton agar (Biolab) plates to give rise to uniform bacterial lawn. Afterward, antibiotic discs were positioned on the plates, and were incubated at 37^{o}C for 24 hours. Thereafter, the discs were observed for zones of inhibition, and the diameter of the zones was recorded (Cheesebrough, 2006). Antibiotics panels used were primarily broad spectrum antibiotics (BSAs) namely, ofloxacin (10 µg), streptomycin (30 µg), cotrimoxazole (30 µg), chloramphenicol (30 µg), sparfloxacin (10 µg), ciprofloxacin (10 µg), amoxicillin (30 µg), amoxicillin/clavulanate potassium (30 µg), gentamicin (10 µg), erythromycin (15 µg), pefloxacin (30 µg), ampiclox (30 µg), cefuroxime(25 µg), and ceftriaxone (25 µg). The susceptibility profiles of the isolates were defined according to the CLSI standards (CLSI, 2017). Intermediate-resistance was regarded as resistance. An isolate was designated as multidrug resistant (MDR) if found resistant to ≥3 structurally different antibiotic classes (Oteo, 2002).

2.7.2. Multiple Broad Spectrum Antibiotics Resistance Indexing of Surface Water Isolates

Multiple broad spectrum antibiotic resistance index (MBSARI) was estimated as the ratio of the number of BSAs to which a surface water isolate was resistant to the sum of all BSAs panel to which the isolate was subjected.

2.7.3. Determination of Metal Resistance

The isolates (wild and cured) were subjected to different metal concentrations (0.001 to 1M) according to De Magalhaes *et al.* (2008) and Mohania *et al.* (2008). Briefly, appropriate dilutions (0.001M, 0.01M, 0.1M and 1M) of iron, zinc, cadmium and nickel were incorporated into Mueller-Hinton agar plates. Then, the isolates were inoculated and incubated in the plates at 37^{o}C for 48 hours. Finally, the test isolates were observed for metal susceptibility.

2.8. Data Analysis

The data obtained were subjected to analysis of variance (ANOVA), and treatment means were separated using Duncan's New Multiple Range Test at 95 % confidence level with the aid of SPSS (version 23).

3. Results

The correlations between plasmids and multidrug and metal resistance in surface water isolates were investigated. The outcome of 16S rRNA sequencing analysis revealed the identities of the isolates as *Proteus mirabilis*, *Alcaligenes. faecalis*, *Alcaligenes faecalis faecalis*, *Bacillus cereus*, *Stenotrophomonas acidaminiphila*, *Proteus penneri*, and *Lysinibacillus macrolide* sunder the accession numbers KY345400 to KY345404from the sites. Figure 1 represents the plasmid profile obtained after gel electrophoresis. *P. mirabilis* has a plasmid of 2kb pair, *A. faecalis* has a plasmid of 9kb pair, *Serratia* sp. has 2kb pair, *L. macroides* has a plasmid of 9kb, *B. cereus* has two plasmids of 4kb and 6kb pair. *S. acidaminiphila*, *A. faecalis faecalis* and *P. prennei* showed no band after electrophoresis indicating the absence of plasmid.

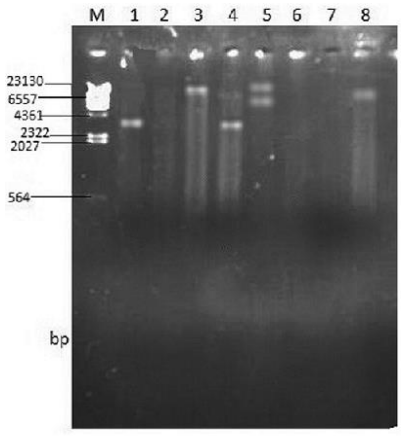

Figure 1. Gel plate showing the Plasmid profile of bacterial isolates before curing (1 – 8 = *P. mirabilis, S. acidaminiphila, A. faecalis, Serratia sp., B. cereus, P. penneri, A. faecalis faecalis* and *L. macroide*)

Figure 2 represents the plasmid analysis after the curing procedure. It was observed that there was no visible band showing that the bacteria have been effectively cured.

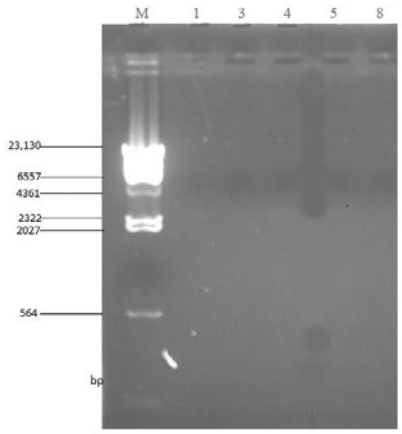

Figure 2. Gel plate showing the Plasmid profile of bacterial isolates after curing (1 – 8 = *P. mirabilis, S. acidaminiphila, A. faecalis, Serratia spp., B. cereus, P. penneri, A. faecalis faecalis* and *L. macroide*)

The antibiogram patterns of the wild and subsequent plasmid-cured isolates are presented in Tables 1 and 2. It showed that some of the microorganisms were multidrug resistant as they exhibited varying degrees of resistance to the antibiotics as follows:- *A. faecalis* (50 %), *P. mirabilis* (57.1 %), *Proteus penneri* (42.85 %), *B. cereus* (28.57 %), *A. faecalis faecalis* (42.86 %), *Serratia* sp. (64.3 %) and *L. macroides* (64.3 %); to the fourteen antibiotics tested and after the curing, *P. mirabilis* lost all its resistance, *A. faecalis* resistance to the antibiotics was reduced to 14.3 %, *P. penneri* retained its resistance, *B. cereus* resistance to the antibiotics was reduced to 21.4%, *A. faecalis faecalis* retained its resistance, *Serratia* sp. resistance to the antibiotics was reduced to 35.7 % and *L. macriodes* resistance to the antibiotics was reduced to 35.7%. The results of the metal resistance test shown in Tables 4 and 5 indicate that there was a decline in the growth of the organisms as concentrations of the metals increased in contrast to the situation in the control i.e., 0.0M of the metals, whereas there was a profuse/heavy growth by all

the organisms except for *Proteus mirabilis* that did not grow at 0.0M cadmium. At 0.001M to 0.1M of nickel concentration, there was growth of all the microorganisms. There was heavy growth of all the microorganisms at a concentration of 0.001 to 0.01M of zinc; however, *B. cereus* still exhibited heavy growth at a 0.1M concentration of zinc. All organisms resisted iron to a concentration of 0.01M at varying degree as expressed by the magnitude of their growth. For the cadmium metal, the organisms showed resistance at a minimal concentration of 0M. After the curing, *A. faecalis* and *P. mirabilis* lost all ability to resist metals; *Bacillus cereus* also retained its ability to resist the metals it previously resisted except cadmium at the same concentration before the curing.

The freshwater isolates foreshadowed a grave and higher risk in terms of multiple broad spectrum antibiotic resistance indices. The MBSARI (%) varied with the species from *B. cereus* (35.7), *A. faecalis* (42.9), *P. mirabilis* (50.0), *P. penneri* (57.1), *S. acidiminiphila* (57.1), *A. faecalis faecalis* (71.4), *L. macroides* (71.4) to *Serratia* sp (78.6) before the curing as shown in Table 3.

Table 1. Antibiotics sensitivity of bacterial isolates before the curing

Antibiotics	*P. mirabilis*	*B. cereus*	*P. penneri*	*Alcaligenes faecalis*	*S. acidiminiphila*	*Alcaligenes faecalis faecalis*	*Serratia* sp.	*L. macroides*
Perfloxacin	23±1.5	23±1.5	0±0.0	21±0.5	23±0.5	18±1.0	0±0.0	0±1.0
Erythromycin	0±0.00	18±1.5	0±0.5	13±1.0	22±2.0	10±1.5	0±1.0	22±1.5
Cotrimoxazole	0±0.00	20±2.0	0±1.5	0±5.0	0±0.0	0±0.0	0±4.0	0±1.0
Streptomycin.	0±0.0	22±0.5	0±1.0	13±4.5	0±1.5	0±0.0	0±1.5	0±0.0
Ciproflaxin	25±2.0	23±0.0	16±3.0	20±2.0	20±2.0	24±2.0	19±0.0	0±0.0
Ceftriaxone	18±2.5	19±1.0	12±4.0	15±2.5	20±0.0	23±0.0	0±0.0	22±2.0
Amoxicillin	21±1.5	22±0.0	19±1.5	16±3.0	0±0.0	0±0.0	14±1.5	0±0.0
Cefuroxime	0±0.5	0±0.0	11±1.5	0±0.0	0±1.0	12±0.0	0±1.0	18±1.5
Ampliclox	0±0.0	0±0.0	0±0.0	0±0.0	0±1.5	8±2.5	0±0.0	24±2.5
Gentamycin	0±0.0	0±0.0	10±1.5	0±1.5	0±2.5	0±0.0	0±0.0	0±1.0
Ofloxacin	18±0.5	18±0.5	0±1.5	0±0.0	18±1.5	16±0.0	15±1.5	0±1.5
Sparfloxacin	16±1.5	20±1.5	20±2.0	11±0.0	20±2.0	21±1.5	12±0.0	0±0.0
Amoxicillin/clavu-lanate potassium	0±0.0	0±0.5	23±0.0	0±1.5	0±1.5	0±0.0	0±0.0	0±0.0
Chloramphenicol	18±0.0	0±1.0	22±0.5	0±2.0	14±2.0	0±0.0	0±1.5	8±0.5

Table 2. Antibiotics sensitivity of the five bacterial isolates with plasmids after the curing

Antibiotics	*P. mirabilis*	*B. cereus*	*Alcaligene faecalis*	*Serratia* sp.	*L. macroides*
Perfloxacin	23±0.0	23±1.0	21±1.0	21±1.0	18±0.0
Erythromycin	15±4.0	18±0.0	14±1.0	16±0.5	20±1.0
Cotrimoxazole	20±1.5	23±0.5	17±0.0	0±1.5	0±0.0
Streptomycin	20±3.0	23±1.0	20±0.5	0±0.5	12±0.5
Ciproflaxin	25±1.0	23±0.0	20±0.0	21±0.0	21±1.0
Ceftriaxone	20±1.5	19±0.5	20±1.5	18±3.5	22±1.0
Amoxicillin	21±1.0	22±1.5	16±1.0	18±1.0	0±0.5
Cefuroxime	16±3.5	0±0.0	18±0.5	16±1.0	18±0.5
Ampliclox	21±0.0	0±0.0	0±2.0	0±0.0	24±0.5
Gentamycin	20±1.5	0±0.0	0±0.0	0±0.5	0±0.0
Ofloxacin	18±1.0	18±0.5	9±0.5	15±2.0	0±0.0
Sparfloxacin	16±2.0	20±0.0	11±0.0	12±0.0	17±1.0
Amoxicillin/ Clavulanate potassium	15±0.0	0±0.5	16±1.5	18±0.0	0±0.0
Chloramphenicol	15±1.0	18±1.0	18±0.0	0±0.0	12±0.5

Table 3. Multiple broad spectrum antibiotic resistance index (MBSARI) of surface water bacteria

Bacteria	MBSARI	MBSARI (%)
P. mirabilis	0.500	50.0
B. cereus	0.357	35.7
P. penneri	0.571	57.1
A. faecalis	0.429	42.9
S. acidiminiphila	0.571	57.1
A. faecalisfaecalis	0.714	71.4
L. macroides	0.714	71.4
Serratia sp.	0.786	78.6

Table 4. Resistance of bacterial isolates to heavy metals

Heavy	Bacterial isolates	Heavy metal concentration			
		0.001M	0.01M	0.1M	1M
Nickel	*P. mirabilis*	+	--	--	--
	A.faecalis faecalis	++	++	--	--
	A. faecalis	+++	+	--	--
	S. acidaminiphila	++	++	--	--
	P. penneri	++	+	--	--
	B. cereus	+++	++	--	--
	L.macroides	+++	++	--	--
	Serratiasp	+++	++	--	--
Cadmium	*P. mirabilis*	+	--	--	--
	A. faecalis faecalis	++	++	--	--
	A. faecalis	+	+	--	--
	S. acidaminiphila	+	+	--	--
	P. penneri	++	+	--	--
	B. cereus	+	+	--	--
	L.macroides	++	+	--	--
	Serratiasp	++	+	--	--
Zinc	*P. mirabilis*	+++	+++	--	--
	A. faecalis faecalis	+++	+++	--	--
	A. faecalis	+++	+++	--	--
	S. acidaminiphila	+++	+++	--	--
	P. penneri	+++	+++	--	--
	B. cereus	+++	+++	++	--
	L.macroides	+++	+++	--	--
	Serratiasp	+++	+++	--	--
Iron	*P. mirabilis*	+++	+++	--	--
	A. faecalis faecalis	+++	++	--	--
	A. faecalis	+++	++	--	--
	S. acidaminiphila	++	+	--	--
	P. penneri	+++	+++	--	--
	B. cereus	+++	+++	--	--
	L.macroides	+++	+++	--	--
	*Serratia*sp	+++	+++	--	--

Keys +++ = Heavy growth, ++ = Moderate growth, + = Scanty growth, -- = No growth

Table 5. Resistance of bacterial isolates to heavy metals.

Heavy	Bacterial isolates	Heavy metal concentration			
		0.001M	0.01M	0.1M	1M
Nickel	*P. mirabilis*	--	--	--	--
	A. faecalis	--	--	--	--
	B. cereus	++	+	--	--
	L.macroides	--	--	--	--
	*Serratia*sp	++	+	--	--
Cadmium	*P. mirabilis*	--	--	--	--
	A. faecalis	+			
	B. cereus	--	--	--	--
	L. macrolides	--	--	--	--
	Serratiasp	--	--	--	--
Zinc	*P. mirabilis*	++	+	--	--
	A. faecalis	--	--	--	--
	B. cereus	--	--	--	--
	L. macrolides	+	+	--	--
	*Serratia*sp				
Iron	*P. mirabilis*	--	--	--	--
	A. faecalis	++	++	--	--
	B. cereus	+	+	--	--
	L.macroides	+	+	--	--
	*Serratia*sp	++	++	--	--

Keys +++ = Heavy growth, ++ = Moderate growth, + = Scanty growth, -- = No growth

4. Discussion

This study identified bacterial isolates obtained from Onyearubulem and FUTA streams in Akure. The correlation between multidrug and metal resistance were determined and further profiled for plasmids and plasmid curing was carried out. The isolates obtained and identified by the 16S rRNA sequencing included *Proteus mirabilis, Alcaligenes faecalis, A. faecalis* sub *faecalis, Bacillus cereus, Stenotrophomonas acidaminiphila, Proteus penneri* and *Lysinibacillus macroides*. Pindi *et al*., (2013) reported the isolation of these bacteria from drinking water samples as potential pathogenic microorganisms. The isolation of *Alcaligenes faecalis faecalis, Proteus penneri, Proteus mirabilis* is in agreement with the investigation of Scot *et al.* (2003) and Nigoyi *et al.* (2005) who reported faecal contaminants as the major sources of microbial entry into surface waters. Okonko *et al.* (2008) also reported that the presence of coliforms in the water samples generally suggests that a certain portion of the water may have been contaminated by faeces of either human or animal origin. The presence of *B. cereus, L. macroides* and *S. acidaminiphila* also supports the fact that non-point source of water pollution such as run-offs from the surrounding environment is a cause of pollution of the surface water (Petersen *et al.*, 2005, Donovan *et al.*, 2008). These microorganisms are known to be majorly associated with the environment close to the surface water; the proximity of the surface water and the environment allows for the run-off of water which allows for the transfer of microorganisms from the environment to the water;

Kampfer *et al.* (2013) had previously isolated members of *Lysinibacillus* from surface water.

The isolates showed varying degrees of antimicrobial resistance to the twelve conventional antibiotics tested. *A. faecalis faecalis* showed 42.85 % resistance, *S. acidaminiphilia* showed 50 % resistance, *Proteus mirabilis* showed 57.1 % resistance, *Proteus penneri* showed 50 % resistance, *B. cereus* showed 35.7 %, *A. faecalis* showed 50 % resistance, *Lysinibacillus macrolides* showed 64.3 % resistance, and *Serratia* sp. showed 64.3 % resistance to the antibiotics tested.

During this study, it was observed that a higher level of multidrug resistance was exhibited by the Gram negative bacteria than their Gram positive counterpart; this is in consonance with Ronald *et al.*, (2002) who found out that a high level of antibiotic resistance was seen in all studied Gram negative bacteria isolated from the rivers of United States.

The resistance level of microorganisms to heavy metals was measured by turbidity in the growth media. Badar *et al*. (2000) stated that the microbial load decreases with an increase in the concentration of heavy metals indicating the toxic effect of the heavy metals on the growth of microorganisms. On all the heavy metals (Cd, Ni, Zn and Fe) tested there was no observable growth of microorganisms at the highest concentration (1.0M). This supports the theory stated by Konopka *et al.* (1999) that a lethal toxic effect is usually observed when bacteria are exposed to a high concentration of metal ions as their resistance mechanisms do not offer protection at extremely high levels of free metal ions. The resistance pattern indicated that among the four experimented heavy metals, the bacteria isolates showed highest resistance to zinc and iron metals; *B. cereus* was resistant to 0.1M zinc metal, whereas isolates had the highest level of susceptibility on cadmium metal, as the lowest growth was observed at 0.01M concentration. This may be because Zn and iron offered low toxicity to the isolates while Ni and Cd expressed more toxicity. However, according to

Malik and Jaiswal, (2000), there are no current acceptable concentrations of metal ions which can be used to distinguish metal resistant and metal sensitive bacteria.

The plasmid profile of the bacterial isolates revealed the presence of plasmids in all the isolates except *S. acidaminiphila, A. faecalis faecalis* and *P. prennei.* This finding supports Ronald *et al.* (2002) who reported that more than 40 % of multiple antibiotic-resistant bacteria have at least one plasmid.

P. penneri showed no band after electrophoresis, which shows that the resistance displayed by the organism to antibiotics and heavy metals was chromosomal mediated. This is in agreement with a previous finding by Kishore, (2012) that *P. penneri* strains are multiple-drug resistant based on their ability to chromosomally induce the production of β-lactamase Hug A.

The absence of plasmids showed that the resistance exhibited by *S. acidaminiphila* is chromosomally induced. This is in agreement with previous studies (Ryan *et al*., 2009 and Shivani *et al*., 2016) which indicated that *S. acidaminiphila* have great genetic versatility and adaptation, and this enhances their ability to survive in varying conditions such as surface water polluted with industrial wastewater.

The resistance of *P. mirabilis* to gentamycin, chloramphenicol, and streptomycin has been reported by Feglo *et al*. (2010). Similar results were also reported by Newman et *al*., (2006) and Bashawan and Shafey, (2013).

After the curing, the isolates showed different susceptibility patterns to the antibiotics it resisted before the curing whereas some maintained their resistance.

Proteus mirabilis lost all its resistances to the antibiotics it previously resisted, it also lost all its resistance to cadmium, nickel and iron, but still showed scanty growth on plates containing zinc. This indicates that the gene responsible for its resistance to the antibiotics and metal are plasmid borne. It has been reported by several studies (Bashawan and Shafey, 2013, Yao and Moellering, 1999) which are in consonance with this study that *Proteus mirabilis* has intrinsic resistance to nitrofurantoin and tetracycline, but is generally susceptible to the ampicillin, amoxicillin, gentamicin and ciprofloxacin. However, resistances to these aforementioned antibiotics occur when these agents have been misused. The loss of multidrug resistance ability of *P. mirabilis* after the curing has also been reported by several investigators. Dharmadhikari and Peshwe (2009), Adeniyi *et al*., (2006), Stankowska *et al*., (2008) and Yah *et al*., (2007) all reported *Proteus mirabilis* carrying plasmids and inferred that the plasmids were able to move genetic antibiotic resistant materials among various bacterial strains, and contribute to the overall pathogenic potential of the bacteria, and when the plasmids were cured, the plasmids were lost as well as the resistances to β-lactam antibiotics as ampicillin, amoxicillin, and gentamycin. *A. faecalis* lost its resistance to cotrimoxazole, cefuroxime, ofloxacin and chloramphenicol, and also lost all its ability to resist all metals it previously resisted. It, however, maintained its resistance to ampliclox and gentamycin. This result indicates that the gene responsible for its resistance to cotrimoxazole, cefuroxime, ofloxacin and chloramphenicol and also zinc, nickel, iron and cadmium metal is plasmid-mediated; however, resistance to ampliclox and gentamycin are chromosomally-mediated.

After the plasmid curing, *B. cereus* showed no considerable decrease in antibiotics' resistance, however, it became susceptible to previously resisted chloramphenicol. A considerable change in metal resistance, namely zinc and cadmium was observed. The significant loss of *Bacillus cereus* tolerance to the metals indicates that its ability to resist metals was plasmid-mediated. This is in agreement with the findings of Amalesh *et al.*, (2012). The investigation of Amalesh *et al.*, (2012) had earlier reported the presence of plasmids in *B. cereus* isolated from industrial waste water, in their report, the presence of plasmids in the bacteria mostly enhanced their metal resistance, while the antibiotics resistance was relatively low.

After the curing *L. macriodes* was susceptible to streptomycin, ciproflaxin, sparfloxacin, perfloxacin and chloramphenicol, but it retained its resistance to cotrimoxazole, amoxicillin, gentamycin, ofloxacin and amoxicillin/ clavulanate potassium. It also lost its resistance to the cadmium and nickel metals. This result indicates that the resistance to cotrimoxazole, amoxicillin, gentamycin, ofloxacin and amoxicillin/clavulanate potassium exhibited by *L. macriodes* was chromosomally-

mediated, whereas its resistance to streptomycin, ciproflaxin, sparfloxacin, perfloxacin and chloramphenicol together with the resistance to metals were mediated by resistant genes borne on the plasmid.

Serratia sp. lost its resistance to perfloxacin, erythromycin, ceftriaxone and cefuroxime, but retained resistance to cotrimoxazole, streptomycin, ampliclox, chloramphenicol and gentamycin. It also lost the ability to resist cadmium; however it retained its resistance to the zinc, nickel and iron metals. This result indicates that the genes responsible for resistances to cotrimoxazole, streptomycin, ampliclox, chloramphenicol and gentamycin together with that responsible for resistance to nickel, iron and zinc metal are chromosomally- based.

The resistances to metal and antibiotics and subsequent loss of resistance after the curing procedure exhibited by these organisms confirm the correlation between metal ions and antibiotics' resistance. This has also been reported by several other researchers on bacterial species from different sources (Rajbanshi, 2008, Mgbemena, 2012). Many have speculated and even shown this to be as a result of the likelihood that resistance genes to both antibiotics and heavy metals could be closely located on the same plasmid in the bacteria, and are thus more likely to be transferred together in the environment (Nies, 1999). Tsai, (2006) reported that many antibiotic resistant genes are located on mobile genetic elements (e.g., plasmids, transposons and integrons), some of which are easily exchanged among phylogenetically distant bacteria. Many of these mobile genetic elements encode resistance to multiple antibiotics, heavy metals and other compounds. This phenomenon is further aided by the process of conjugation where a bacterium in the population serves as donor, and transfers the gene to the other recipients in the population.

5. Conclusion

Microbes have adapted to tolerate the presence of metals and actions of certain antimicrobials. As a result of this, they have become insensitive to the action of antimicrobials and the presence of metals through various mechanisms. Their potential to resist metals not only ends by being able to absorb and use them for growth, but also extends to their untapped potentials to clean up metal-contaminated sites. Other implications of this resistance are not beneficial as the increase in antimicrobials portends future health risks and challenges. Hence, there is a need to manipulate them to either harness or destroy their resistance potentials.

Acknowledgements

We are grateful to the Department of Microbiology, Federal University of Technology, Akure, Nigeria for the facilities used for this study. We are also grateful to FOWN Biotechnology Limited, Lagos, Nigeria for the molecular studies performed in their laboratory.

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JJBS

Jordan Journal of Biological Sciences

Volume 11, Number 4,September 2018
ISSN 1995-6673
Pages 369 - 374

Correlations of Serum Chemerin and Visfatin with other Biochemical Parameters in Iraqi Individuals with Metabolic Syndrome and Type Two Diabetes Mellitus

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Received December 11, 2017; Revised January 18, 2018; Accepted January 31, 2018

Abstract

Chemerin and visfatin are bioactive molecules that regulate numerous physiological functions such as energy equilibrium, insulin action, inflammatory response and vascular homeostasis. The objective of this study is to determine the correlation between serum chemerin and visfatin with other biochemical parameters in Iraqi patients with metabolic syndrome (MetS) and type 2 diabetes mellitus (T2DM). Twenty-two participants with MetS, forty-four with T2DM and twenty-two healthy subjects were recruited in this study. Serum concentrations of chemerin and visfatin, hs-CRP, fasting plasma glucose (FPG), fasting serum insulin (FSI), lipid profile for all participants were measured. Their correlations with the anthropometric parameters, insulin resistance and MetS parameters were specified. The results revealed that the MetS group has the highest serum levels of chemerin and hs-CRP compared to T2DM and the control groups (151.77±10.43 ng/mL, 129.36±5.03 ng/mL and 63.98±14.74ng/mL respectively) with significant difference of (P<0.001) and (8.1±1.1mg/L, 7.96±1.18mg/L and 4.55±2.31mg/L respectively). Visfatin serum concentration was higher in the T2DM group than that of the MetS and control group (63.71±8.30ng/mL, 56.03±10.58 ng/mL and 52.46±14.05 ng/mL respectively). Both of these two adipokines were found to be correlated with some parameters. Moreover, no correlation was found between the two proteins. From the obtained results we concluded that the assessment of chemerin and visfatin levels and their relation to some metabolic parameters can help to identify subjects who are more susceptible to the cardiovascular disease (CVD risk.

Keywords: Chemerin, Visfatin, Metabolic syndrome, T2DM.

1. Introduction

The metabolic syndrome (MetS) refers to a cluster of related metabolic abnormalities, such as central obesity, hypertension, dyslipidemia, hyperglycemia, and insulin resistance (Martínez and Andriantsitohaina, 2017). The central obesity and insulin resistance in particular were known as causative factors for the metabolic syndrome (Srikanthan *et al*., 2016). The increase in the metabolic syndrome prevalence is associated with the increasing overweight, obesity, and physical inactivity (Martínez and Andriantsitohaina, 2017). For the purpose of the diagnosis of MetS, three out of five abnormal conditions should exist in the patient (fasting hyperglycemia, high blood pressure, hypertriglyceridemia, low high-density lipoprotein cholesterol levels, and central obesity) (Alberti *et al.*, 2006). Several studies showed that the MetS is associated with an approximate two-fold increased risk of developing cardiovascular disease (CVD) and a five-fold increased risk for incident type two diabetes mellitus over the next five to ten years (Cornier *et al*., 2008).

Diabetes mellitus type 2 is a combination of disorders that are characterized by elevated blood levels of glucose and are associated with microvascular and macrovascular complications (Zaccardi *et al*., 2015). The endogenous insulin deficiency or resistance to the insulin action in muscle, fat and liver in addition to the inadequate response by the pancreatic beta cells result in hyperglycemia (Wolfs *et al*., 2009). The adipose tissue is defined as an active endocrine organ that secretes an enormous number of bioactive mediators (adipokines) that signal to the organs of metabolic importance including the brain, liver, skeletal muscle, and the immune system thereby modulating hemostasis, blood pressure, glucose and lipid metabolism, inflammation and atherosclerosis (Bozaoglu *et al.*, 2007). These adipokines include: adiponectin, leptin, omentin, resistin, and interleukin-6, tumor necrosis factor-α, visfatin, vaspin and chemerin (Yan *et al.*, 2012).

Chemerin is one of these bioactive mediators defined as a multifunctional peptide involved in the glucose and lipid metabolism. Raised levels of this peptide have been associated with insulin resistance and systemic inflammation (Fatima *et al*., 2015). This protein is highly expressed in liver and the white adipose tissue. Increased

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levels of chemerin have been observed in mice and humans that are obese and have T2DM (Bozaoglu *et al*., 2007). The metabolic effect of chemerin in diabetes is the dysregulation of insulin and glucose metabolism (Lehrke *et al*., 2009).

Visfatin is the adipokine that exerts insulin-mimetic effects, which stimulate muscle and adipocyte glucose transport, and inhibit the hepatocyte glucose creation. It is expressed mainly in the visceral adipose tissues as well as in the human liver, muscles, and macrophages. Visfatin prompts the production of cytokines, such as interleukin-6 and tumor necrosis factor-α, in the human leukocytes. Its plasma level rises during chronic inflammatory conditions such as psoriasis, arthritis, and obesity (Adghate, 2008). Also, it increases in T2DM, which is characterized by insulin resistance (IR), and is usually observed in gestational diabetes mellitus (GDM) (Lewandowski *et al*., 2007). The relations between visfatin and metabolic syndrome, such as insulin resistance and dyslipidemia in humans have recently been studied, and numerous aspects of these relations are unknown. However, Mohammadi *et al.* found that high circulating levels of visfatin could be in healthy relations with cardiovascular risk factors, insulin resistance status and adiponectin in diabetic patients (Mohammadi *et al*., 2011).

The purpose of this study is to determine the serum levels of chemerin and visfatin in Iraqi individuals with the metabolic syndrome and type two diabetic patients. Furthermore, it is designed to find the correlation between these two adipokines; chemerin and visfatin as well as between them and other anthropometric and biochemical parameters.

2. Materials and Methods

2.1. Study Subjects

This cross sectional study was conducted in cooperation with the National Diabetes Center for Treatment and Research at Al-Mustansiriya University in Baghdad city, Republic of Iraq. Eighty-eight subjects were enrolled in this study: forty-four patients with T2DM (twenty-seven males and seventeen females) aged between twenty and seventy years; and BMI of 28.35±3.68 kg/m^2, twenty-two patients with MetS (thirteen males and nine females) aged between twenty and sixty years; BMI of 38.71±6.24 kg/m^2, and twenty-two healthy participants (with no family history of diabetes, high cholesterol, hypertension or other diseases) (fourteen males and eight females) aged between twenty and sixty years; BMI of 24.77±3.48 kg/m^2.

Participants with T2DM were included in the study according to the World Health Organization criteria (Alberti and Zimmet, 1998). The diagnosis of MetS was based on the global consensus of MetS according to the 2005 International Diabetes Federation (Alberti *et al.*, 2006). The patients must have central obesity (BMI is >30kg/m²), and any two out of the rest four factors of MetS diagnosis: 1) Elevated Triglycerides ≥ 150 mg/dL (or specific treatment for this lipid abnormality). 2) Decreased HDL- cholesterol value (< 40 mg/dL in males and < 50 mg/dL in females) or specific treatment for this lipid abnormality. 3) Raised blood pressure; systolic blood pressure ≥ 130 mm Hg or diastolic blood pressure ≥ 85 mmHg or (having been diagnosed with hypertension and were treated). 4) Elevated fasting plasma glucose (FPG; ≥ 110 mg/dl) or have been diagnosed with type II diabetes. The exclusion criteria included patients with type one diabetic mellitus, T2DM taking insulin as hypoglycemic therapy, acromegaly, chronic liver and kidney diseases. The study was approved by the Human Research Ethics Committee of the Center, and informed agreements were obtained from each patient.

2.2. Blood Sample Collection

Ten milliliters of venous blood were collected after ten-twelve hours of fasting from each subject and then were then divided into two aliquots. For the first aliquot (2 ml), EDTA containing tube was used for the assessment of fasting plasma glucose, while for the second aliquot (8 ml), biochemistry tubes with a gel separator were used. After thirty minutes of an incubation period, the samples were centrifuged (at 1500 × g for fifteen minutes). A portion of the obtained serum was used for the estimation of lipid profile and uric acid. The second portion of the serum used for the subsequent assay of fasting serum insulin, hs-CRP, chemerin and visfatin was stored at -20^{o}C.

2.3. Anthropometric Measurements

Weight (Kg), height (cm), and waist circumference (cm) were measured for all the participants. The body mass index (BMI) was calculated by dividing the weight (in kg) over the height square (m^2), waist to height ratio (WHtR) as well as body fat percentage (BF %) was calculated according to the following equations:

WHtR = waist (cm)/ height (cm)

BF% = (1.20 x BMI) + (0.23 x age) - (10.8 x sex) - 5.4 …. (Deurenberg *et al*., 1991)

Sex: male=1, female=2

2.4. Blood Pressure Measurements

Mercury sphygmomanometer was used to measure the Systolic blood pressure (SBP) and diastolic blood pressure (DBP) (mmHg) in a sitting position. The mean arterial pressure (MAP) was calculated from these measurements according to the equation below:

MAP = DBP + (SBP-DBP)/3 …… (Bouchra *et al*., 2005)

2.5. Laboratory Tests

The estimation of plasma glucose was performed by a glucose oxidase method. The total serum cholesterol was estimated by enzymatic colorimetric tests with cholesterol esterase and cholesterol oxidase, while the serum triglycerides evaluation was done by the enzymatic colorimetric tests with glycerol phosphate oxidase. The HDL-cholesterol was evaluated after precipitation of the apolipoprotein B-containing lipoproteins with phosphotungstic acid. The low-density lipoprotein cholesterol was calculated by the Friedewald formula (Friedewald *et al*., 1972). The atherogenic index of plasma (AIP) was calculated using the equation:

AIP =Log (Tg / HDL-C) ….. (Dobiášová and Frohlich, 2001)

The homeostasis model assessment of insulin resistance (HOMA-IR) was calculated from fasting insulin and glucose by the equation bellow (Mattews *et al*., 1985):

HOMA-IR = insulin (micro units per milliliter) × glucose (mg/dl) / 405

The insulin resistance was defined at the cutoff point corresponding to HOMA-IR ≥ 3.8 (Shirai, 2004). The fasting serum insulin level was estimated by ELISA using the commercially available ELISA kit (Monobind Inc., U.S.A). High sensitivity C-reactive protein was measured by bioactive diagnostic (Germany) ELISA kit. Human chemerin serum level was measured using the commercially available chemerin ELISA assays kit (Ray Biotechnology Company, U.S.A). The visfatin serum level was estimated also by using Ray Bio Visfatin Enzyme Immunoassay (EIA) Kit (U.S.A). All ELISA procedures were carried out according to the manufacturer's instructions.

2.6. Statistical Analysis

In order to analyze the data statistically, the IBM SPSS software package (version 22.0) was used. The variables were reported as means ± standard deviation. The groups were compared by using one way ANOVA and post hoc Tukey test. The correlations between the serum of visfatin, chemerin and other variables were detected using the Pearson's correlation analysis, with a *P* value of <0.05 indicating the statistically significant difference.

3. Result

Our study consisted of eighty-eight participants divided into three groups: T2DM group (n=44), MetS group (n=22) and the control group (n=22). The general anthropometric, clinical and biochemical features of the participants are represented in Table 1. The Statistical ANOVA and post hoc test showed that there is a significant difference in age between the "control and T2DM" groups as well as "T2DM and MetS" groups at ($P < 0.001$), but there wasn't any significant difference between the control and MetS groups. Waist circumference & waist to height ratio showed that there was a high significant difference in T2DM and MetS groups compared with the control group at ($P < 0.001$), while there wasn't any significant difference in the comparison of T2DM & MetS groups together. BMI was significantly higher in the metabolic syndrome group and T2DM group compared to the control group at ($P < 0.001$), as well as BF %.

Fasting plasma glucose (FPG) in the T2DM group was significantly higher than Mets and control groups at ($P < 0.001$), while FSI and HOMA-IR in Mets group were significantly higher than T2DM and control groups at ($P < 0.001$). By comparing the means of total serum cholesterol of the three groups, it is found that there were no significant differences between them.

Table 1. Clinical and biochemical characteristics of the study

Factor	Control N=22	T2DM N=44	MetSN=22	*P*
Age (y)	33.41±9.55	56.68±8.24 $^{**a, c}$	37.59±10.15 $^{** c}$	<0.001
Sex (M/F)	14M/8F	27M/17F	13M/9F	____
WC (cm)	82.27±7.80	97.64±14.63 ^{**a}	103.0±10.57 ^{**b}	<0.001
BMI(kg/m^2)	24.77±3.48	28.35±3.68 $^{**a, c}$	38.71±6.24 $^{**b, c}$	<0.001
BF%	25.05±7.56	35.23±7.84 $^{**a, c}$	43.16±9.02 $^{**b, c}$	<0.001
WHtR	0.48±0.03	0.58±0.09 ^{**a}	0.62±0.06 ^{**b}	<0.001
FPG(mg/dL)	87.95±10.81	171.09±53.76 ^{**a,c}	109.64±7.16 $^{** c}$	<0.001
FSI(μlU/mL)	7.55±1.86	13.13±1.92 ^{**a,c}	19.25±1.09 $^{**b, c}$	<0.001
HOMA-IR (%)	0.97±0.25	1.96±0.34 ^{**a,c}	2.56±0.16 $^{**b, c}$	<0.001
TC(mg/dL)	168.90±36.96	181.70±43.84	128.27±33.13	0.259
TG(mg/dL)	77.18±29.26	148.59±93.32 ^{**a,c}	84.31±40.40 $^{** c}$	0.001
HDL-C(mg/dL)	47.38±12.09	37.38±9.86 $^{**a, *c}$	44±8.83 ^{*c}	0.001
LDL-C(mg/dL)	106.06±38.14	116±39.78	128±32.29	0.160
VLDL-C(mg/dL)	15.02±6.31	29.70±18.72 ^{**a,c}	16.86±7.90 $^{** c}$	0.001
SBP(mmHg)	118.9±16.3	141.8±23.5 ^{**a,c}	125±19.5 $^{** c}$	<0.001
DBP(mmHg)	74.5±10.7	81.5±13.8	74.09±11.6	0.030
MAP(mmHg)	89.3±11.5	101.6±16.2 ^{**a,*c}	91±13.8 $^{* c}$	0.002
AIP	0.11±0.05	0.55±0.28 ^{**a,c}	0.26±0.19 $^{** c}$	<0.001
hs-CRP(mg/L)	4.55±2.31	7.96±1.18 ^{**a}	8.1±1.1 ^{**b}	<0.001
Chemerin(ng/mL)	63.98±14.74	129.36±5.03 ^{**a,c}	151.77±10.43 ^{**b,c}	<0.001
Visfatin(ng/mL)	52.46±14.05	63.71±8.30 ^{**a,*c}	56.03±10.58 $^{* c}$	<0.001

Results were expressed as mean ± SD, ANOVA test was used for the purpose of comparison between the three groups. *P< 0.05 is significant, **P<0.01 is highly significant. a refers to the significant differences between control and T2DM. b refers to the significant differences between controls and MetS. c refers to the significant differences between for T2DM and MetS. T2DM =Type 2 diabetes mellitus, MetS=Metabolic syndrome, WC= waist circumference, BMI=body mass index, BF %=body fat percentage, WHtR=waist to height ratio, FPG=fasting plasma glucose, HOMA-IR= homeostasis model of assessment-insulin resistance, TC=total cholesterol, TG= triglycerides, HDL= high-density lipoprotein, LDL= low-density lipoprotein; VLDL= very low-density lipoprotein, SBP= systolic blood pressure, DBP=diastolic blood pressure, MAP= mean arterial pressure, AIP= atherogenic index of plasma, hs-CRP=high-sensitivity C-reactive protein.

Serum TG in the T2DM group was significantly higher compared to that of both the control and the metabolic syndrome groups ($P < 0.01$), as well as VLDL-C, while mean of serum HDL-C was significantly higher in the control group compared to the patients groups ($P< 0.01$). However, the mean of serum LDL-C was higher in the MetS group compared to the T2DM and control group. The means of Systolic blood pressure (SBP), diastolic blood pressure (DBP), arterial pressure (MAP) and atherogenic index of plasma (AIP) revealed a significant increase in the diabetic group compared with the metabolic syndrome and control groups. Figure 1 illustrates that chemerin serum levels in the MetS group were higher than in the T2DM and control group, as well as hs-CRP (Figure 2). In contrast, visfatin serum levels in the T2DM group were higher than those of the Mets and control groups, Figure (3).

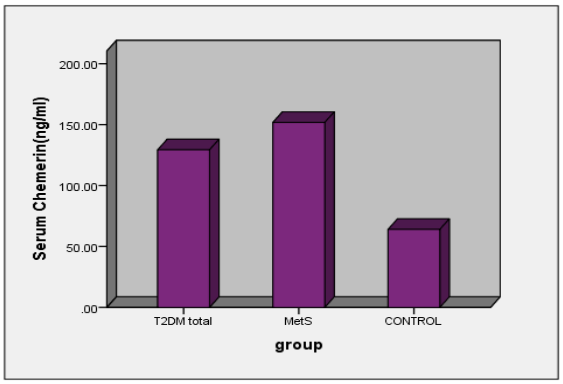

Figure 1. Chemerin serum levels of the three groups

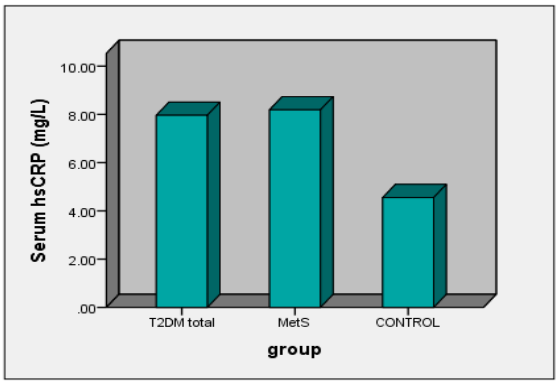

Figure 2. hs-CRP serum Levels of the three groups

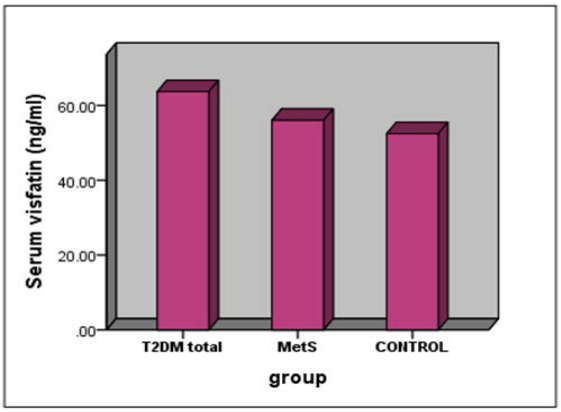

Figure 3: Visfatin serum levels of the three groups

Table 2 shows the Pearson correlation coefficient of serum chemerin and serum visfatin in the diabetic group. Based on the results, the chemerin serum level had a positive correlation with BMI (P =0.011), BF% (P = 0.049), WHtR (P = 0.036) and hs-CRP (P = 0.002), while the visfatin serum level had a positive correlation with BF % (P = 0.041), and is negatively correlated with triglycerides (P=< 0.001), VLDL (P=< 0.001) and AIP (P = 0.01). According to Table 3, chemerin serum levels in the MetS group had a positive correlation with gender (P = 0.001), BF % (P= 0.032), triglycerides (P= 0.001), VLDL (P=0.001) and AIP (P= 0.004). Visfatin serum levels were positively correlated with BMI (P= 0.011), BF % (P= 0.049) and WHtR (P= 0.036), while being negatively correlated with gender (P = 0.038).

Table 2. Correlations between chemerin and visfatin serum levels with anthropometric and laboratory data of T2DM group (n=44)

Parameter	Serum Chemerin		Serum visfatin	
	R	*P*	*R*	*P*
Age(y)	0.055	0.723	0.242	0.114
Sex (M/F)	0.102	0.510	0.251	0.101
WC(cm)	0.211	0.180	-0.172	0.256
BMI	0.380	0.011	0.143	0.353
BF%	0.299	0.049	0.310	0.041
WHtR	0.318	0.036	-0.103	0.507
FPG(mg/dL)	0.047	0.761	0.022	0.888
Insulin(µlU/mL)	0.215	0.162	0.293	0.054
HOMA-IR (%)	0.189	0.219	0.239	0.119
TC(mg/dL)	0.147	0.342	-0.157	0.307
TG(mg/dL)	0.085	0.584	-0.509	< 0.001
HDL(mg/dL)	0.098	0.525	0.182	0.238
LDL(mg/dL)	0.094	0.544	0.020	0.895
VLDL(mg/dL)	0.086	0.578	-0.507	< 0.001
SBP(mmHg)	0.139	0.369	0.003	0.987
DBP(mmHg)	-0.030	0.849	-0.041	0.790
MAP(mmHg)	0.050	0.746	-0.022	0.886
AIP	0.044	0.779	-0.382	0.01
hs-CRP(mg/L)	0.449	0.002	0.294	0.053
Chemerin(ng/mL)	1	-	0.066	0.673
Visfatin(ng/mL)	0.066	0.673	1	-

r, Pearson coefficient. *Statistically significant at $P \leqslant 0.05$. **highly significant at $P \leqslant 0.01$. WC= waist circumference, BMI=body mass index, BF%=body fat percentage, WHtR=waist to height ratio, FPG=fasting plasma glucose, HOMA-IR= homeostasis model of assessment-insulin resistance, TC=total cholesterol, TG= triglycerides, HDL= high-density lipoprotein, LDL= low-density lipoprotein, VLDL= very low-density lipoprotein, SBP= systolic blood pressure, DBP=diastolic blood pressure, MAP= mean arterial pressure, AIP= atherogenic index of plasma, hs-CRP=high-sensitivity C-reactive protein.

Table 3. Correlations between chemerin and visfatin serum levels and anthropometric and laboratory data of MetS group (n=22)

Parameter	Serum Chemerin		Serum Visfatin	
	R	*P*	*R*	*P*
Age(y)	0.080	0.725	0.038	0.868
Sex(M/F)	0.648	0.001	-0.445	0.038
Waist(cm)	0.160	0.478	0.211	0.180
BMI	0.060	0.790	0.380	0.011
BF%	0.458	0.032	0.299	0.049
WHtR	0.333	0.130	0.318	0.036
FPG(mg/dL)	-0.003	0.991	-0.077	0.733
Insulin(μlU/mL)	-0.132	0.558	-0.238	0.287
HOMA-IR(%)	-0.110	0.625	-0.222	0.320
TC(mg/dL)	0.388	0.075	0.147	0.342
TG(mg/dL)	0.672	0.001	0.085	0.584
HDL(mg/dL)	0.093	0.680	0.098	0.525
LDL(mg/dL)	0.199	0.374	0.094	0.544
VLDL(mg/dL)	0.661	0.001	0.086	0.578
SBP(mmHg)	-0.042	0.852	0.025	0.913
DBP(mmHg)	-0.235	0.293	0.021	0.927
MAP(mmHg)	-0.151	0.501	0.023	0.918
AIP	0.587	0.004	0.044	0.779
hs-CRP(mg/L)	-0.011	0.961	0.233	0.297
Chemerin (ng/mL)	1	-	-0.364	0.096
Visfatin (ng/mL)	-0.364	0.096	1	-

r, Pearson coefficient. *Statistically significant at $P \leqslant 0.05$. **highly significant at $P \leqslant 0.01$. WC= waist circumference, BMI=body mass index, BF%=body fat percentage, WHtR=waist to height ratio, FPG=fasting plasma glucose, HOMA-IR= homeostasis model of assessment-insulin resistance, TC=total cholesterol, TG= triglycerides, HDL= high-density lipoprotein; LDL= low-density lipoprotein; VLDL= very low-density lipoprotein, SBP= systolic blood pressure, DBP=diastolic blood pressure, MAP= mean arterial pressure, AIP= atherogenic index of plasma, hs-CRP=high-sensitivity C-reactive protein.

4. Discussion

The present study aimed to assess the levels of serum chemerin and visfatin in patients with T2DM and MetS. It is aimed also to examine the correlations between these two adipokines. The results of the study reveal that serum chemerin levels were higher in the subjects with MetS than those of the diabetic patients and healthy individuals. In contrast, serum visfatin levels in the T2DM group were higher than the Mets and control groups. The statistical tests showed that there was no correlation between chemerin and visfatin among the studied groups. Previous studies revealed that the serum level of chemerin is higher in individuals with MetS compared to healthy individuals (Jialal *et al.*, 2013, Chu *et al*., 2012 and Bozaoglu *et al.*, 2009). In addition, Bozaoglu *et al*. found that the serum level of chemerin was associated with many indicators of this syndrome such as triglyceride, HDL, and fasting insulin level among a Mexican-American population. They also found that the level of this adipokine was higher in obese than lean individuals (Bozaoglu *et al*., 2009). It is well- known that obesity is one of the most important outcomes of modern lifestyles which continually raise the risk for many diseases development.

BMI and waist circumference, body fat percentage are markers for obesity. As obesity is accompanied with increased body fat and this adipokine is produced by adipose tissues, the increase in cell count and adipose tissue results in an increase in the production of this adipokine (Zanganeh *et al*., 2016). Numerous studies indicate that chemerin affects glucose homeostasis, and could be the link between increased adipose tissue mass/fatty liver disease and obesity-related metabolic and inflammatory diseases. Chemerin was found to be associated with many components of the MetS, including BMI, triglycerides, high-density lipoprotein cholesterol, and hypertension, and also with systemic markers of inflammation, such as high sensitivity C-reactive protein (hs-CRP), interleukin-6 (Chakaroun *et al*., 2012). These results are in agreement with the results of the current study which showed a positive correlation between chemerin and some of the MetS parameters in both patient groups, such as BF %, BMI, triglycerides, VLDL, hs-CRP and AIP (Tables 2 & 3). As illustrated in Figure 1, serum levels of chemerin in the T2DM patients were higher compared to the healthy subjects and such result came in concordance with that of El-Mesallamy et al. and Tarik et al. (El-Mesallamy *et al*., 2011 and Tarek *et al*., 2013), who found that chemerin serum levels in patients with type two diabetics were higher than that in non-diabetic healthy individuals. Similarly, Susana et al. found that circulating chemerin concentrations were elevated in diabetic patients (Susana *et al.*, 2014).

Visfatin is the adipokine that has insulin-mimetic effects and stimulates the muscle and adipocyte glucose transport, and inhibits the hepatocyte glucose creation (Samiha *et al.*, 2013). Diabetic patients have higher levels of serum visfatin compared to MetS and healthy individuals, as shown in Figure 3. Increased visfatin levels in the T2DM patients are independent on obesity and insulin resistance and are mainly determined by levels of fasting glucose and triglycerides (Esteghamati *et al*., 2011). Previous studies found that visfatin levels were higher in patients with T2DM compared to controls (Dogru *et al*., 2007 and Samiha *et al*., 2013). This study revealed that visfatin, in the T2DM group, was not correlated with BMI and WHtR, whereas it was correlated positively with BF% and negatively with TG, AIP and VLDL. In the patients with MetS, visfatin appeared to be correlated positively with BMI and WHtR, and was negatively correlated with sex, disimilar to chemerin which showed a positive correlation with sex as illustrated in Tables 2 & 3 respectively. Both of these adipokines have a positive correlation with body fat percentage. However, in the MetS group, chemerin had a positive correlation with TG, VLDL and AIP.

Many studies showed that there is a strong association between obesity and the metabolic syndrome so that the accumulation of visceral fat has an essential role in metabolic syndrome, cardiovascular disease and obesity-related disorders such as diabetes mellitus, hyperlipidemia and hypertension. The secretion of numerous adipokines by the adipose tissue may be the major mechanisms in these lifestyle-related diseases (Matsuzawa, 2006). Ahmed et al. found that visfatin may be implicated in diabetes pathogenesis, and plays an important role in the development of metabolic syndrome (Ahmed *et al.*, 2015). Recent studies showed that the measurement of serum

levels of chemerin can be effective in the diagnosis of metabolic syndrome (Zanganeh *et al.*, 2016). Lachine et al. concluded that serum level of chemerin correlates with cardio-metabolic disease, with a significant association between chemerin serum concentration and the severity of CAD in Egyptian patients with T2DM (Lachine *et al*., 2016).

5. Conclusion

From the obtained results we conclud that visfatin and chemerin may contribute to the development of insulin resistance and diabetes mellitus. Furthermore, the assessment of chemerin and visfatin levels and their correlations with some metabolic syndrome parameters can help to identify subjects who are most susceptible to the CVD risk.

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JJBS

Volume 11, Number 4,September 2018
ISSN 1995-6673
Pages 375 - 380

Jordan Journal of Biological Sciences

Growth, Maturity and Form Factor of Mola Carplet (*Amblypharyngodon mola*) from the Ganges River, Northwestern Bangladesh

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Received October 19, 2017; Revised February 6, 2018; Accepted February 15, 2018

Abstract

The current study presents the first complete and comprehensive description of population structure, growth pattern (length-weight relationships, LWRs; length-length relationships, LLRs), maturity (size at first sexual maturity; L_m), form factor ($a_{3.0}$) and natural mortality (M_W) of *Amblypharyngodon mola* (Hamilton, 1822) in the Ganges River, northwestern Bangladesh. Additionally, asymptotic weight (W_α), $a_{3.0}$, L_m and M_W of this fish species from different water bodies worldwide were calculated. Seasonal samples of *A. mola* were collected from the Ganges River, northwestern Bangladesh during November 2015 to October 2016 from the fishers' catch using cast net (mesh size ranges: 1.5 - 2.0 cm). The total length (TL) was measured to the nearest 0.1 cm using digital slide calipers, and the total body weight (BW) was measured using an electronic balance with 0.1 g accuracy for each individual. The growth pattern was estimated through LWR as $BW= a*TL^b$, where *a* and *b* are regression parameters. Also, $a_{3.0}$ was calculated using the equation: $a_{3.0} = 10^{\log a\text{-}s(b\text{-}3)}$, where *a* and *b* are regression parameters of LWRs and s = -1.358, is the regression slope of log *a vs. b*. Furthermore, L_m of *A. mola* was calculated using the empirical equation, log (L_m) = -0.1189 + 0.9157 * log (L_{max}), where L_{max} = maximum observed TL. A total of 308 individuals of *A. mola* were analyzed, where minimum and maximum TL was 3.9 cm and 8.1 cm, respectively, and BW was 0.5g and 5.8 g, correspondingly. The highest number (49.00 %) of its population stands at 6.00 cm size group. The *b* value of LWR indicated positive allometric growth, the $a_{3.0}$ was 0.0129, the L_m was 5.16 cm in TL and M_w was 1.75 year^{-1} of *A. mola* in the Ganges River, northwestern Bangladesh. These findings can be very effective for the sustainable management of this fish species in the Ganges River and its ecosystems.

Keywords: *Amblypharyngodon mola*, Growth pattern, form factor, Size at sexual maturity, Ganges River, Bangladesh.

1. Introduction

Amblypharyngodon mola (Cyprinidae), commonly known as Mola Carplet, is widely distributed in Asian countries including Bangladesh, India, Myanmar and Pakistan (Talwar and Jhingran, 1991). This species is a popular food fish mainly in Indian sub-continent due to its high nutritional values (Saha *et al*., 2009) of a high protein, vitamin and mineral content (Mazumder *et al*., 2008). According to Rahman (1989), *A. mola* is extensively found in rivers, canals, *beel*s, ponds, and inundated fields of Bangladesh. Even though this fish species is categorized as a least concern in Bangladesh and globally (IUCN Bangladesh, 2015; IUCN, 2017), unfortunately the natural populations are declining due to the reckless fishing, habitat destruction (Hossain *et al*., 2017a), pollution and other ecological changes to their territory (Hossain *et al*., 2015; Hossen *et al*., 2016).

Information on the population structure of *A. mola* is needed for the appropriate management and the initiation of conservation measures of this important species in the Ganges River. Growth of fishes i.e. length–weight relationships (LWRs), length–length relationships (LLRs) are the most important biological parameters for the management and conservation of the natural populations (Sarkar *et al*., 2009; Muchlisin *et al*., 2010; Hossen *et al*., 2017). Additionally, form factor ($a_{3.0}$) is used to verify whether the body shape of a given species is notably different from others **(**Froese, 2006). Moreover, the size at first sexual maturity is very significant to find out the factors that affect the spawning size of a population (Hossain *et al*., 2013, 2017b; Elahi *et al*., 2017). Entropy on length-frequency distributions (LFDs) (Hossain *et al*.,

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2006, 2012), length-weight relationships (LWRs) (Hossain *et al*., 2016a, b, c, 2017c, d), form factor ($a_{3.0}$) (Hossain *et al*., 2013), and the size at first sexual maturity (L_m) (Hossain *et al*., 2016d) for different fish species in the Indian sub-continent are well documented. To the best of the authors' knowledge, there are no earlier studies on the population structure, growth, maturity, and form factor of *A. mola* from the Ganges River, NW Bangladesh. However, some studies on other aspects of this species from different water bodies have been done including Azadi and Mamun (2004), Mondal and Kaviraj (2013), Ahamed et al. (2017a, 2017b) etc. Therefore, this study presents the first reference on the population structure (length-frequency distributions; LFDs), growth (LWRs, LLRs), size at the first sexual maturity (L_m), form factor ($a_{3.0}$) and natural mortality (M_w) of *A. mola* from the Ganges River, NW Bangladesh.

2. Materials and Methods

In the current study, a total of 308 individuals of *A. mola* were collected seasonally from the Ganges, River of northwestern Bangladesh during November, 2015 to October, 2016 from the fishers' catch. The samples were caught using various types of traditional fishing gears i.e., cast net (mesh size ranges: 1.5 - 2.5 cm), gill net (mesh size ranges: 1.5–2.0 cm), and square lift net (mesh size: ~2.0 cm). The fresh samples (dead fish) were instantly chilled in ice on site and preserved with 10 % buffered formalin after arrival in the laboratory.

The fish were identified up-to species level through morphometric and meristic characteristics according to Rahman (1989) and Fishbase (Froese and Pauly, 2016). The total body weight (BW) of each individual was weighed using an electronic balance with a 0.01 g accuracy. Different linear dimensions i.e. lengths (Total length, TL; Fork length, FL; Standard length, SL) were taken to the nearest 0.01 cm using digital slide calipers.

The growth pattern was estimated through LWR with the equation: $BW = a*L^b$, where W is the body weight (BW, g) and L is the different lengths in cm. The regression parameters a and b were calculated by linear regression analyses based on natural logarithms: ln (W) = ln (a) + b ln(L). Moreover, 95 % confidence limit (CL) of a and b and the co-efficient of determination (r^2) were estimated. Extreme outliers were removed from the regression analyses according to Froese (2006). A t-test was used to confirm whether the b values obtained in the linear regressions were significantly different from the isometric value (b = 3), (Sokal and Rohlf 1987). The LLRs were estimated by linear regression analysis (Hossain *et al*., 2006).

The form factor ($a_{3.0}$) was calculated using the equation given by Froese (2006) as: $a_{3.0} = 10^{\log a-s(b-3)}$, where a and b are regression parameters of LWR (TL *vs.* BW) and s = -1.358, is the regression slope of log a *vs.* b.

The size at first sexual maturity (L_m) of *A. mola* in the Ganges River was calculated using the empirical equation, log (L_m) = -0.1189 + 0.9157 * log (L_{max}) (Binohlan and Froese, 2009) based on the maximum observed length. Additionally, the asymptotic weight (W_α) was determined through LWR using the asymptotic length (L_α) = 10.47 cm (Azadi and Mamun, 2009) for each population. Also to estimate the $a_{3.0}$ in worldwide water bodies, the regression parameter a and b for LWRs of A. mola from different water bodies were obtained from the available literature through the Fish Base and / or the Google search. Furthermore, the maximum lengths of this species were obtained from the available literature to estimate the L_m in different water bodies worldwide.

The M_W of *A. mola* was calculated using the model, M_W =1.92 year^{-1} *(W)$^{-0.25}$ (Peterson and Wroblewski, 1984), where M_W = Natural mortality at mass W, and W = $a*L^b$, a and b are the regression parameters of LWR.

Statistical analyses were performed using Microsoft® Excel-add-in-DDXL and Graph Pad Prism 6.5 software. All statistical analyses were considered significant at 5 % ($p < 0.05$).

3. Results

A total of 308 specimens of *A. mola* were collected from the Ganges River, NW Bangladesh during this study. Table 1 illustrates the descriptive statistics on length and weight measurements with mean values and their 95 % confidence level. The LFDs of *A. mola* showed that TL varied between 3.9 cm and 8.1 cm. The maximum population stands at 6.0 cm to 7.0 cm TL size group (Figure 1). The regression parameters a and b of the LWR, and their 95 % confidence limits, and the coefficients of determination (r^2) and growth type are given in Table 2 and Figure 2. The calculated b values of the LWRs indicated positive allometric growth. All LWRs were highly significant ($P < 0.001$), with r^2 values being greater than 0.953. In addition, the LLRs along with regression parameters p and q, and the coefficient of determination (r^2) are presented in Table 3 and Figure. 3. All LLRs were highly significant ($P < 0.001$), with most coefficients of determination values being >0.971. Furthermore, the calculated W_α, $a_{3.0}$, L_m and M_W of this fish species from different water bodies worldwide are presented in Table 4. The present study reveals that M_W for the population of *A. mola* was 1.75 year^{-1} in the Ganges River, NW Bangladesh (Figure 4).

Table 1. Descriptive statistics on the length (cm) and weight (g) measurements of the *Amblypharyngodon mola* (Hamilton, 1822) (n = 308) in the Ganges River, northwestern Bangladesh.

Measurements	Min	Max	Mean ± SD	95% CL
TL (Total length)	3.9	8.1	5.41 ± 0.77	5.33-5.50
SL (Standard length)	3.0	6.3	4.20 ±0.60	4.13-4.26
FL (Fork length)	3.4	7.1	4.67 ± 0.67	4.60- 4.75
BW (body weight)	0.5	5.8	1.65 ±0.87	1.56- 1.75

Min, minimum; Max, maximum; SD, standard deviation; CL, confidence limit for mean values.

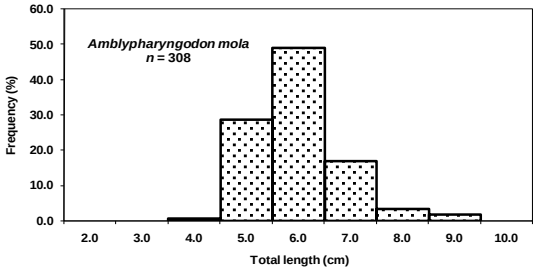

Figure 1. Total length frequency distribution of *A. mola* from the Ganges River, northwestern Bangladesh

Table 2. Descriptive statistics and estimated parameters of the length-weight relationships of the *Amblypharyngodon mola* (Hamilton, 1822) in the Ganges River, northwestern Bangladesh.

Equation	*N*	*a*	*B*	95% CL of *a*	95% CL of *b*	r^2	GT
$BW=a*TL^b$		0.0067	3.21	0.0059 -0.0077	3.14- 3.29	0.957	A^+
$BW=a*SL^b$	308	0.0159	3.18	0.0143-0.0177	3.11 to 3.26	0.960	A^+
$BW=a*FL^b$		0.0114	3.18	0.0101-0.0129	3.10 -3.26	0.953	A^+

n, sample number; C, combined sex; BW, body weight; TL, total length; SL, Standard length; FL, Fork length; *a* and *b* are regression parameters and GT, growth type; A^+=positive allometric

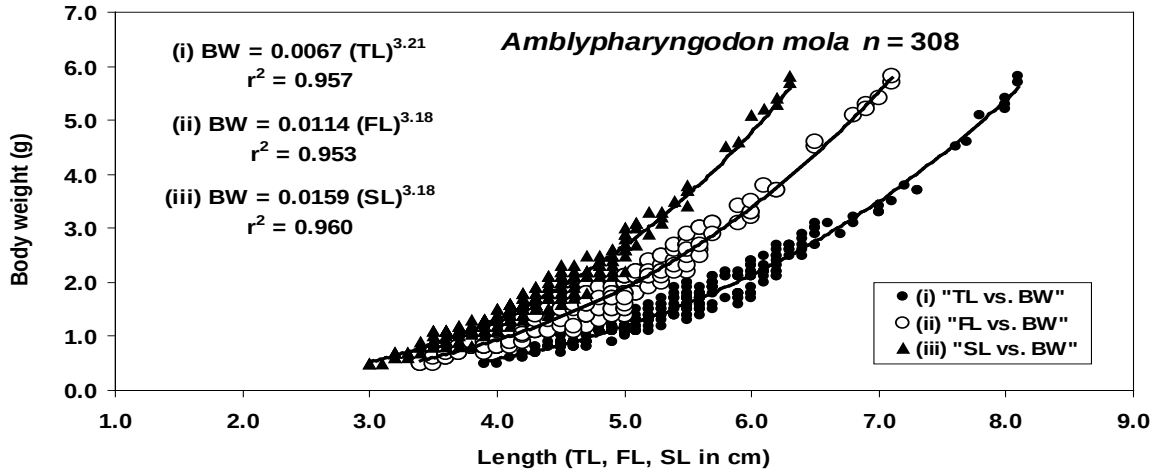

Figure 2. Length-weight relationships of *Amblypharyngodon mola* from the Ganges River, northwestern Bangladesh

Table 3. Descriptive statistics and estimated parameters of the length-length relationships of the *Amblypharyngodon mola* (Hamilton, 1822) in the Ganges River, northwestern Bangladesh.

Equation	*p*	*Q*	95% CL of *p*	95% CL of *q*	r^2
TL=*p*+*q*(SL)	0.1009	1.27	0.0006- 0.2013	1.24-1.29	0.973
TL=*p*+*q*(FL)	0.1050	1.14	0.0134-0.1964	1.11-1.16	0.978
SL=*p*+*q*(FL)	0.0740	0.88	-0.0072 to 0.1551	0.87-0.90	0.971

n, Sample number; C, combined; *p*, Intercept; *q*, Slope ; TL, Total Length; SL, Standard Length; r^2, Coefficient of Determination

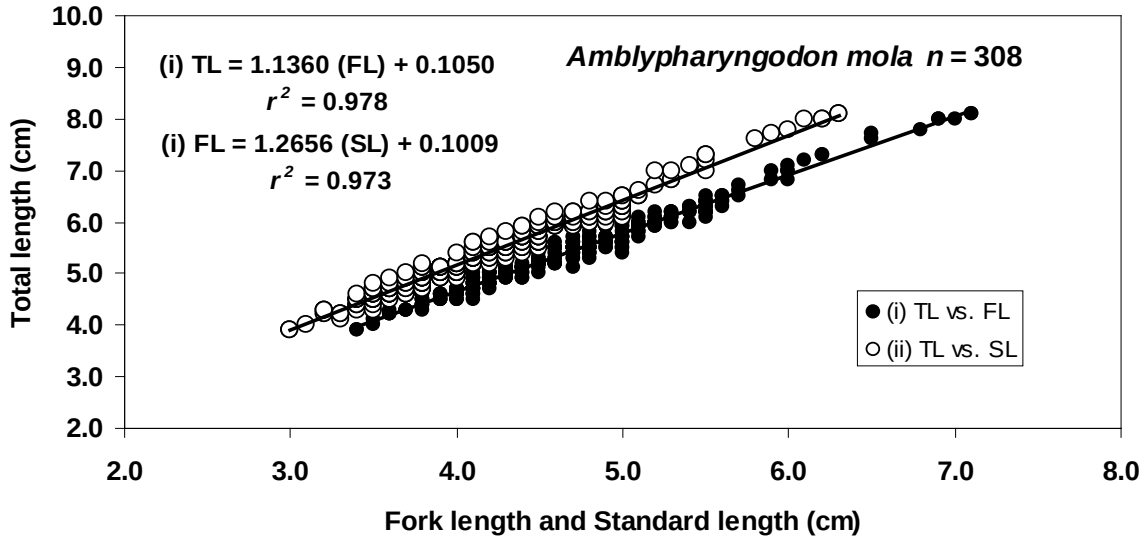

Figure 3. Length-weight relationships of *Amblypharyngodon mola* from the Ganges River, northwestern Bangladesh

Table 4. The calculated size at sexual maturity and form factor $a_{3.0}=10^{\log a - s(b-3)}$ of *Amblypharyngodon mola* (Hamilton, 1822) in different water bodies worldwide.

Water body	TL_{max}	W_α	Regression Parameter		References	$a_{3.0}$	L_m (95% CL of L_m)	M_w
			A	*b*				
Garjan beel, Assam	7.6	12.60	0.0053	3.31	Baishya *et al*. (2010)	0.0138	4.87 (4.00-5.98)	1.27
Ganges lower region	5.9*	28.06	0.0109	3.34	Hossain *et al*. (2009)	0.0316	3.86 (3.20-4.71)	1.33
Mathabhanga River	7.0	16.15	0.0055	3.40	Hossain *et al*. (2006)	0.0190	4.52 (3.72-5.53)	1.35
Garjan beel, Assam	8.3	17.79	0.0037	3.61	Baishya *et al*. (2010)	0.0250	5.28 (4.32-6.50)	1.07
Payra River, Bangladesh	5.8*	5.76	0.0065	2.89	Ahamed *et al*. (2017a)	0.1494	3.80 (3.16-4.63)	1.85
Wetlands of Assam	9.0	14.19	0.1678	2.87	Devi and Das (2017)	0.1118	5.69 (4.64-7.02)	0.62
Atrai & Bramhaputra River	6.2	1.14	0.009	2.06	Islam *et al*. (2017)	0.0005	4.04 (3.35-4.93)	2.29
Ganges River	-		0.1097	1.92	Sarkar *et al*. (2013)	0.0037	-	
Gomti River	-		0.0132	1.82	Sarkar *et al*. (2013)	0.0003	-	
Rapti River	-		0.1097	1.91	Sarkar *et al*. (2013)	0.0036	-	
South 24 Parganas, India	8.7		-	-	Pal *et al*. (2014)	-	5.51 (4.50-6.80)	
Wetland of Balarampur, Baruipur, West Bengal	8.9		-	-	Gupta and Banerjee (2015)	-	5.63 (4.59-6.95)	
Ganges River	**8.1**	**12.59**	**0.0067**	**3.21**	**Present study**	**0.0129**	**5.16 (4.23-6.35)**	**1.75**

TL, total length; *standard length; max, maximum; W_α = asymptotic weight; *a* and *b* are regression parameters of length-weight relationships; $a_{3.0}$, form factor; L_m, size at first sexual maturity; CL, confidence limit for mean values; M_W, natural mortality.

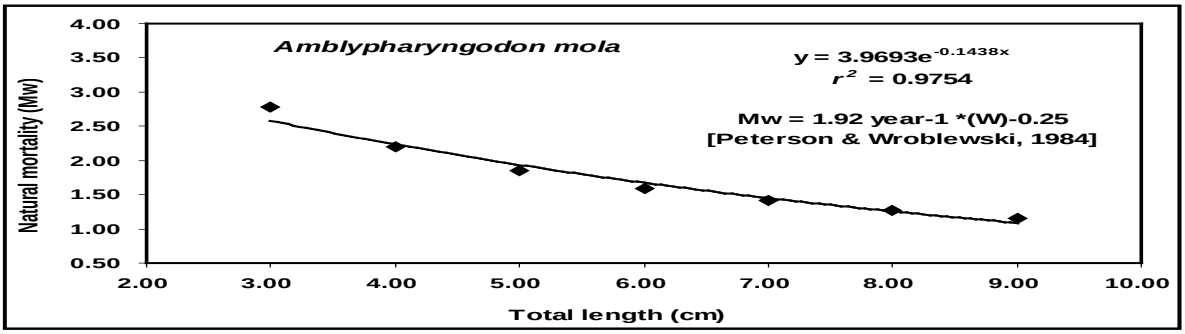

Figure 4. The natural mortality (M_W) of *Amblypharyngodon mola* from the Ganges River, northwestern Bangladesh

4. Discussion

During this study, a large number of specimens of *A. mola* were collected with various body sizes. However, it was not possible to collect < 8.1 cm TL, which can be attributed to the selectivity of fishing gears/ mesh (Hossain *et al*., 2017a). The present study observed the maximum TL of *A. mola* as 8.1 cm which is lower than the maximum recorded length of 15.0 cm (Ahmad, 1953). According to Rahman (1989) the maximum length of this fish species as 9.0 cm. Bhuiyan (1964) recorded the maximum TL as 8.0 cm which is lower than the present study. The variations in the recorded maximum TL of *A. mola* in different waters can be attributed to the nonexistence of bigger-sized individuals in the populations in fishing grounds/ areas (Hossain *et al.,* 2016d). In addition, the variations in the fishing gear used and the selectivity on the target species may greatly influence the size distribution of the caught individuals which resulted in highly biased estimations of various population parameters including the maximum size (Hossain *et al.*, 2017b, Azad *et al*., 2018).

The present study revealed that the calculated *b* value (3.21) for TL *vs.* BW lies between 2.50 and 3.50 (Froese, 2006). In earlier studies, Hossain (2010) and Hossain *et al*., (2009) recorded the regression parameter '*b*' as 3.76 and 3.34, respectively for *A. mola* from the Ganges River, NW Bangladesh. Additionally, Hossain *et al*. (2006) also recorded positive allometric growth in *A. mola* (*b* = 3.40) in the Mathabhanga River, southwestern Bangladesh, which are similar with the present findings. Gogoi and Goswami (2014) recorded the '*b*' value of LWR for combined sex of *A. mola* from the Jorhat district of India as 2.97 indicating negative allometric growth. This finding is not compatible with the result of the present study. However, the *b* values may vary in the same species due to the mixture of one or more factors including variations of growth in different body parts, sex, physiology, preservation methods, and differences in the observed length ranges of the specimens collected (Tesch, 1971; Hossen *et al*., 2016, 2018; Nawer *et al*., 2017), which were excluded during this study. All LLRs were highly correlated, and were compared with the available literatures.

The $a_{3.0}$ of this fish species was within the limits reported by Froese (2006), and Hossain *et al*. (2012). In addition, the form factor ($a_{3.0}$) using available *a* and *b* regression parameters of LWRs in ten different water bodies worldwide have been calculated. The $a_{3.0}$ can assess whether the body shape of individuals in a given population or species is considerably different from others

(Froese, 2006). No references dealing with the $a_{3.0}$ are available in the literature about these species, and therefore the present results provide an important basis for future comparisons.

Studies on size at first sexual maturity (L_m) for *A. mola* from the Ganges River, NW Bangladesh are absent in the literature. Studies dealing with L_m of this species from different regions have been conducted (Suresh *et al.*, 2007; Hoque and Rahman, 2008; Gupta and Banergee, 2013). In this study, The L_m for *A. mola* was 5.16 cm TL, regardless of sex which would be used for the permissible size of catch and for the special awareness in the fisheries management (Lucifora *et al*., 1999). Suresh et al. (2007) found L_m 5.1-5.6 cm and 3.9-4.4 cm whereas Hoque and Rahman (2008) reported 4.8 cm and 5.5 cm for males and females, respectively. Gupta and Banerjee (2013) have documented 5-5.5 cm and 5.5-6 cm for males and females, respectively. The variation on L_m might be due to geographical changes. Additionally, the L_m for *A. mola* from nine different water bodies has been calculated using the maximum length in the available literature, which would be used for conservation regulations in its own habitat.

The calculated W_α in this study was 12.59 g. Hossain et al. (2009) have used juveniles for their study, resulting in an error in the calculated W_α. Additionally, the b value from the study of Sarkar et al. (2013) might be wrong as well because the b values are not between 2.0 to 3.0 (Carlander, 1969). The M_w for the population of A. mola was estimated as 1.75 year^{-1} in the Ganges River, NW Bangladesh. Comparing this value with the calculated M_W of other water bodies, it is found that the value is almost similar with the calculated value of the Payra River, Bangladesh, but is much higher than all the calculated values of Indian waterbodies (Table 4), which may be attributed to the geographical variation.

In conclusion, our study gives valuable information on the population structure, growth pattern (length-weight relationships; length-length relationships), maturity (size at first sexual maturity), form factor and natural mortality of *A. mola*. The results of this study can be very beneficial for further studies in the Ganges River and other water bodies. Furthermore it can serve as a valuable means for stock assessment and a sustainable management of this fish species in the Ganges River and its ecosystems.

Acknowledgements

The authors would like to extend their sincere appreciation to the NATP-2, PIU-BARC, CRG Sub-Project No, 484 for the instrumental and partial funding support, and also to the Ministry of Education, Bangladesh (No. 37.200000.004.003.005.2014-1309/1 (42); Date: 10-08-2014) for providing funding to carry out this research.

Conflicts of interest

The authors declare that there is no conflict of interest regarding the publication of the present paper.

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JJBS
Jordan Journal of Biological Sciences

Volume 11, Number 4,September 2018
ISSN 1995-6673
Pages 381 - 387

Treatment of Aspirin and CCl_4-Induced Hepatic Damage in Rats by the Aqueous Extracts of some Local Plants Collected from Gombe State in Nigeria

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Received January 2, 2018; Revised February 15, 2018; Accepted February 20, 2018

Abstract

In the present study, the capacity of the aqueous extracts of *Senna singueana* (SS), *Nymphaea lotus* (NL), *Cochlospermum planchoni* (CP) and *Acacia nilotica* (AN) as antitoxicants to protect against aspirin and carbon tetrachloride (CCl_4) induced hepatotoxicity in rats was investigated. Ten groups containing three replicates each were used. Biochemical parameters including aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), direct bilirubin (DB), total bilirubin (TB), total protein (TP) and albumin (ALB) were assayed. SS, NL, CP and AN extracts (250 mg/kg) were given daily by gavage to the animals in the groups V to X for fourteen consecutive days to explore the protective effects against aspirin and CCl_4-induced hepatotoxicity. Animals of negative and standard controls (group I and II) respectively received vehicle and vehicle with 2 mL/kg olive oil by subcutaneous injections twice a week for a period of two weeks. Animals of the CCl_4-treated group (group III) and the aspirin-treated group (group IV) respectively received vehicle with 2 mL/kg CCl_4 in olive oil by subcutaneous injections and vehicle with 1 mL/kg aspirin orally twice a week for a period of two weeks. The results obtained were statistically evaluated using One-Way ANOVA followed by Least Significant Difference (LSD) for the parameters found to be statistically significant at $\alpha = 0.05$. Mean serum AST, ALT and ALP, DB and TB levels/activities of the groups III and IV were statistically ($p < 0.05$) higher than those of the controls. DB and TB levels were slightly ($p > 0.05$) higher when compared with the controls, in contrast with the mean serum TP and ALB levels of the groups III and IV that were found to differ statistically ($p < 0.05$) being lower than those of controls. Conversely, mean serum AST, ALT and ALP, DB and TB levels/activities of the groups V to X differ statistically ($p < 0.05$) being lower than those of the groups III and IV except for DB and TB levels that were slightly lower ($p > 0.05$) when compared with the groups III and IV. Mean serum TP and ALB levels of the groups V to X and the controls were found to differ statistically ($p < 0.05$) being higher than those of the groups III and IV. Taken together, the results of this study showed that the SS or NL extracts were found effective as hepatoprotective agents, and the mixture of SS and CP or the mixture of NL and AN extracts significantly antagonized aspirin and CCl_4-induced liver damage in rats in comparison with control values, as evidenced by the biochemical parameters.

Keywords: *Acacia nilotica*, Aspirin, Carbon tetrachloride, *Cochlospermum planchoni*, Hepatotoxicity, *Nymphaea lotus*, *Senna singueana*.

1. Introduction

According to the World Health Organization (WHO), in 2015, 325 million people worldwide have been estimated to be living with the chronic hepatitis infection (Wikipedia, 2017; CDC, 2017). Globally, 1.34 million people died of hepatitis in 2015. The majority of infants (80 – 90 %) infected during the first year of life developed chronic infections. 30-50 % of the children infected before the age of six years developed chronic hepatitis (Wikipedia, 2017; CDC, 2017). Liver is the first major organ to be exposed to ingested toxins due to its portal blood supply. Toxins may be, at least partially, removed

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from the circulation during the first pass, providing protection to other organs while increasing the likelihood of hepatic injury. Liver toxicity is monitored in standard toxicity studies by a range of investigations including clinical biochemical parameters such as enzymes, proteins and lipids (Sharma *et al*., 2012; Jaiswal *et al*., 2015; Gupta *et al*., 2015; Sharma *et al*., 2016). "Hepatitis" refers to the inflammation of the liver which could be a short-term (acute) inflammation or a long-term (chronic) one depending on whether it lasts for less than or more than six months (Wenden & Bernal, 2013). Toxins, chemicals, certain drugs, some diseases, heavy alcohol use, heavy metals and bacterial and viral infections can all cause hepatitis (Sharma *et al*., 2012; Gupta *et al*., 2015; Sharma *et al*., 2016). However, aspirin, carbon tetrachloride (CCl_4) and carbofuran are by far the most essential drugs and chemicals used to induce the oxidative stress not only in the Wistar rats, but in their brain and liver slices as well (Sharma *et al*., 2016;). Hepatitis is also the name of a family of viral infections that affect the liver. Its most common types are Hepatitis A, Hepatitis B, and Hepatitis C (Sharma *et al*., 2012), and to a lesser extent Hepatitis D and Hepatitis E (Sharma *et al*., 2012; CDC, 2017). Perturbations in the activity of the liver function enzymes (LFTs) following the aspirin, carbon tetrachloride (CCl_4) or carbofuran administration in Wistar rats have been extensively studied as previously reported by Sharma *et al*. (2012), Jaiswal *et al*. (2015), Gupta *et al*. (2015), Sharma *et al*. (2016), Gupta *et al*. (2017) and Jaiswal *et al*. (2017). Curcumin and vitamin C have been shown to have protection against carbofuran induced oxidative stress in brain, heart and liver slices in rats (Sharma *et al*., 2016; Gupta *et al*., 2017; Jaiswal *et al*., 2017).

Aspirin is one of the potent non-steroidal anti-inflammatory drugs (NSAIDs) used for the treatment of inflammatory conditions. A high dose of aspirin can cause damage as it impairs the ability of the gastrointestinal mucosa to respond to the injury (Drugs.com, 2014). Aspirin is a chemical that can induce severe liver damage in experimental animals. In 1971, Vane and his coworkers discovered that aspirin causes mucosal damage by interfering with the prostaglandins synthesis. Aspirin acts by inhibiting cyclooxygenases (COX-1 and COX-2), the enzymes that convert arachidonic acid to prostaglandins, thus reducing the PG levels (Vane, 1971). As prostaglandins play a major role in the maintenance of the gastroduodernal defense, their depletion due to aspirin impairs the cytoprotection which results in mucosal injury, erosions and ulceration (Vane & Botting, 2003). Carbon tetrachloride (CCl_4) has been extensively used in animal models to explore chemical toxin-induced hepatic injuries (Sharma *et al*., 2012). The metabolism of CCl_4 catalyzed by liver microsomal cytochrome P450 rapidly overproduces free radicals that deplete hepatic glutathione, and initiate a chain of lipid peroxidation of the hepatocytes membrane (Recknagel *et al*., 1989). This ultimately results in the overproduction of reactive oxygen species (ROS) and hepatocytes injuries (Mohammed *et al*., 2014). Liver damage induced by CCl_4 involves the biotransformation of the free radical derivatives, increased lipid peroxidation, and excessive cell death in the liver tissues (Mohammed *et al*., 2014).

Senna singuena (Delile) Lock, commonly known as "Winter cassia" (English) and "Rumfu" (Hausa) is a widespread plant in the semi-arid parts of tropical Africa. It is usually found in the savanna and is abundantly available where shrubs used to grow, either on the lowlands or the hills. *S. senguena* has an anti-oxidant, anti-inflammatory, anti-dysentric, anti-cancer, anti-fever antipyretic, anti-worms, anti-syphilis, anti-ulcer (leaf/root), and antibacterial activities against both gram positive and negative bacteria (Sepasal, 2006). According to Kawanga and Bosch (2007), and Mebrahton *et al.* (2016), the phytochemical screening results of *S. Singuena* confirmed the presence of Alkaloids, carbohydrate, glycosides, phenols, steroids, tannins and triterpenes. *Nymphaea lotus* L, popularly known as "Water lily" (English) and "Bado" (Hausa) grows in various parts of East Africa and Southeast Asia. It consists of various phenols, tannins, saponins, steroids, proanthocyanidins, flavanols, alkanoids and flavonoids (Madhusudhanan *et al.*, 2011; Afolayan *et al.*, 2013). *N. lotus* is very rich in phytochemicals, and is a good source of natural antioxidants. This may justify its use in the treatment of several diseases affecting humans (Afolayan *et al.*, 2013). *N. nymphaea* has antioxidant, antitumor, sedative, anti-inflammatory, anti-cancer, aphrodisiac, antiviral, antibacterial, and demulcent activities (Madhusudhanan *et al.*, 2011; Afolayan *et al.*, 2013). It is used in traditional medicine systems as an aphrodisiac, astringent, cardiotonic, sedative, demulcent, analgesic, and as anti-inflammatory agent (Madhusudhanan *et al.*, 2011). *Cochlospermum planchoni* Hook.f., variously called "False cotton" (English) and "Rawaya" (Hausa) is a perennial plant with a woody subterranean found in the savannas and the savanna forests. It is found in dried rocky areas (Anaga and Oparah, 2009). *C. planchoni* has alkaloid, phenolics, carbohydrates, glycosides, anthraquinones, saponins, steroidal triterpenes, flavonoids, tannins, cardenolides and dienoloides (Nafiu *et al.*, 2011; Isah *et al.*, 2013). *C. planchoni* possesses antibacterial, antimalarial, antityphoid, anti-hepatobililary infections (black toilets fever) antidiabetic, anti-inflammatory and analgesic activities (Anaga and Oparah, 2009). *Acacia nilotica* (L.) Delile, popularly known as "Tomentosa Babul" (English) and "Bagaruwa" (Hausa) is usually found in the regularly flooded areas. The chemical constituents of *A. nilotica* include alkaloids, flavonoids, glycosides, saponins, tannins, stearic acid, vitamin C, polysaccharides and terpenoids (Amos *et al.*, 1999; Deshpande, 2011). Several bioactive agents have been identified from *A. nilotica* which include: androstene steroid, gallic acid, ellagic acid, kaempferol, naringenin, rutin, lupine, niloticane, umbelliferone catechin, and sitosterol (Lee *et al.*, 2011; Kannan *et al.*, 2013). Previous scientific studies on different parts of *A. nilotica* revealed that the plant has anti-inflammatory, hypoglycemic, antitumor, anti-fungal, antiplatelets aggregation, spasmogenic and vasoconstrictor, antihypertentive, antihepatitis (Lee *et al.*, 2011). Many researches have been previously conducted on the hepatoprotective effects of many medicinal plants, such as the aqueous extracts of *Pterocarpus erinaceus* and *Bauhinia rufescens* by Usman *et al.* (2017); nutraceuticals by Mohammed *et al.* (2014); *Xylopia aethiopica* by Adekeye *et al.* (2014); dandelion by Al-Malki *et al.*

(2013); *Cnicloseous aconititolius* by Saba *et al.* (2010); honey and aloe vera by Adewoga and Sebiomo (2014); *Bauhinia racemosa* by Gupta *et al*. (2004); *Vernonia amygdalina* by Adesanoye *et al.* (2010); among others. However, little or no work has been carried out on the assessment of the hepatoprotective effects of the aqueous extracts of *S. singuena*, *N. lotus*, *C. planchoni* or *A. nilotica* singly or in combination with one another in both aspirin and CCl_4-induced liver damaged rats. This research is designed to investigate these gaps. It is aimed at assessing the hepatoprotective effects of the aqueous extracts of *S. singuenas*, *N. lotus*, *C. planchoni* and *A. nilotica* in albino rats for a period of two weeks. The specific objectives of this research are as follows: 1) Induction of liver damage using aspirin and CCl_4. 2) Assaying the activities/levels of AST, ALT, ALP, TB, DB, TP and Albumin in the experimental groups. 3) Assessing the hepatoprotective effects of SS, NL, CP and AN, by the context of liver function tests (LFTs), following the induction of liver damage using aspirin and CCl_4.

2. Materials and Methods

2.1. Study Area

This research was carried out at the Department of Biological Sciences' Laboratory, Federal University of Kashere in Gombe State, Nigeria. The study was approved by the ethical committee of the University prior to the experimentation, and all the experiments were performed according to the guidelines of the Institutional Animal Ethical Principle.

2.2. Chemicals

All chemicals were of the highest commercially analytical grade, and were obtained from Sigma-Aldrich Co., USA.

2.3. Laboratory Animals

Apparently thirty healthy male and female Wistar rats (*Rattus norvegicus*) with body weights ranging from 152 to 309 g were obtained from the National Veterinary Research Institute, Vom, Plateau State, Nigeria. They were acclimatized for a period of one week in a well-ventilated room, and were housed in a well-ventilated plastic cage maintained under standard laboratory conditions (twelve hours light/dark cycle; 25 – 32 ^oC) prior to experimentation. They were fed with commercial rat chow (Vital Feeds LMT, Plateau State, Nigeria) and sachet water *ad libitium*, and were handled according to the standard protocols.

2.4. Collection of Plant Materials

Fresh leaves of *S. singuena* and roots of *C. planchoni* were collected from Kashere town, Akko Local Government, Gombe State, Nigeria, in December, 2017. Fresh leaves of *N. lotus* were collected from Wuro ibba Dam, Dukul town, Kwami Local Government, Gombe State, Nigeria, in January, 2017. Pod of *A. nilotica* was collected from the Bajoga town, Funakaye Local Government, Gombe State, Nigeria, in January, 2017. The plants were identified and authenticated by the Department of Biological Sciences, Federal University, Kashere, Gombe State – Nigeria. The voucher specimen No. was prepared and deposited at the Herbarium of Federal University of Kashere (FUKH) for reference. A Batch/code number for all the plant samples were issued by Mr Umar Galadima, (Head of Biological laboratory, FUK) as follows: *Senna singueana* leaf; FUKH077, *Nymphaea lotus* leaf; FUKH078, *Cochlospernum planchonmi* root; FUKH076 and *Acacia nilotica* seed; FUKH079.

2.5. Induction of Liver Damage by Aspirin and CCl4

The concentration of aspirin was determined by using the following formula: Wt/ Vol (mg/mL). Each tablet of aspirin was dissolved in three mL of distilled water. A total of thirty-three tablets of aspirin (each 300 mg, 33 tab X 300 mg = 9900 mg) were dissolved in 99 mL of distilled water, making 100 mg/mL. 1 mL/kg was administered orally to the experimental groups. 16 mL of CCl_4 from the stock was dissolved up to 100 mL of olive oil. 2 mL/kg was administered subcutaneously to the experimental groups.

2.6. Experimental Design

A total of thirty Wistar rats were randomly divided into ten groups consisting of three rats each as follows:

Group I: served as negative/normal control that is allowed free access to vital food and water only.

Group II: neutral/standard control: olive oil (2 mL/kg) twice a week (day 0, day 4, day 8, day, 12) for a period of two weeks.

Group III: positive control: CCl_4 (2 mL/kg) dissolved in olive oil, twice a week for a period of two weeks.

Group IV: positive control: Aspirin (1 mL/kg) twice a week for a period of two weeks.

Group V: CCl_4 (2 mL/kg) twice a week for a period of two weeks plus *Senna singueana* (250 mg/kg) daily for two weeks;

Group VI: CCl_4 (2 mL/kg) twice a week for a period of two weeks plus *Nymphaea lotus* (250 mg/kg) daily for two weeks;

Group VII: Aspirin (1 mL/kg) twice a week for a period of two weeks followed by *Senna singueana* (250 mg/kg) daily for two weeks;

Group VIII: Aspirin (1 mL/kg) twice a week for a period of two weeks followed by *Nymphaea lotus* (250 mg/kg) daily for two weeks;

Group IX: CCl_4 (2 mL/kg) twice a week for a period of two weeks followed by *Senna singueana* (250 mg/kg) plus *Cochlospernum planchoni* daily for two weeks;

Group X: Aspirin (1 mL/kg) twice a week for a period of two weeks followed by *Nymphaea lotus* (250 mg/kg) plus *Acacia nilotica* daily for two weeks.

2.7. Preparation of the Plants Aqueous Extracts and Biochemical Analysis

The fresh leaves of *Senna singuena* and *Nymphaea lotus*, roots of *Cochlospermum planchoni*, and the pods of *Acacia nilotica* were washed, air-dried until constant weight was obtained, and were grinded into fine powder using a mortar and pestle. The powder was poured into a Bama bottle for each sample and was labeled. 350 g of each of the powder of *Senna singuena*, *Nymphaea lotus*, *Cochlospermum planchoni* and *Acacia nilotica* was soaked in 1 L (1000 mL) of distilled water, shaken for three minutes and was then allowed to stay for three days (seventy-two hours). The mixtures were filtered with Whatman No. 1 filter paper (25 cm) on the third day. The filtrates were evaporated to dryness using a water bath evaporator at 40 – 50 ^oC which took two weeks in order to obtain the crude extract that was reconstituted up to 200

mL distilled water (Won *et al.*, 2005). At the end of the experiment, the rats were sacrificed twenty-four hours after the last administration of aqueous extracts, and the blood samples were collected into lithium heparin tubes for Liver Function Tests (LFTs). The samples for LFTs were transported to the Biochemistry Laboratory of Gombe State University (GSU) and were centrifuged for ten minutes at 3000 rpm to separate the serum. Sera were carefully separated into clean dry Wassermann tubes by using a Pasteur pipette, and were tested for the activity of Aspartate Aminotransferase (AST) and Alanine Aminotransferase (ALT) (Reitman and Frankel, 1957), and Alkaline Phosphatase (ALP) (Rec, 1972), as well as the level of Direct Bilirubin (DB) and Total Bilirubin (TB) (Jendrassik and Grop, 1938), Total Protein (TP) (Keller, 1984) and Albumin concentration using Randox Kit.

2.8. Statistical Analysis

The results obtained were statistically evaluated using One-Way Analysis of Variance (ANOVA). Differences were considered statistically significant at $p < 0.05$ followed by Least Significant difference (LSD) to determine where the difference among the ten groups containing three rats each actually lies.

3. Results

Table 1 shows the results of mean serum levels/activities of AST, ALT, ALP, TP and ALB of group I to IV. When mean serum levels of AST, ALT, ALP, TP and ALB of group III and IV are compared with controls (group I and group II), the difference is statistically significant ($p< 0.05$).

Mean serum levels of AST, ALT and ALP of group III and IV were found to be statistically ($p < 0.05$) higher when compared with controls (group I and II). Conversely, mean serum levels of TP and ALB of group III and IV were found to differ statistically ($p < 0.05$) being lower than those of controls. Thus, the subcutaneous injection of CCl_4 (group III) or the oral administration of Aspirin (group IV) induced significant elevation ($p < 0.05$) of the ALT, AST and ALP levels, and somehow caused significant reduction ($p < 0.05$) in the TP and ALB levels when compared with controls (group I and II).

The results of Table 2 reveal mean serum levels/activities of AST, ALT, ALP, TP and ALB of the groups I, IV, VII, VIII and X. When mean serum levels of AST, ALT, ALP, TP and ALB of group IV are compared with control, the difference is statistically significant ($p < 0.05$). Mean serum levels of AST and ALP of the groups VII, VIII and X were found to differ statistically ($p < 0.05$) being lower when compared with group IV in contrast to the ALT levels of these groups that were found to differ slightly ($p > 0.05$) being lower when compared with group IV. Conversely, the mean serum levels of TP and ALB of the groups VII, VIII and X were found to differ statistically ($p < 0.05$) being higher than those of group IV. Therefore, the pre-treatment with the SS, NL or a mixture of NL and AN extracts caused significant reduction ($p < 0.05$) in the AST, ALT and ALP activities, and in the same vein induced significant elevation in the TP and ALB levels when compared with the Aspirin-induced group (IV).

Table 1. Serum activities of biochemical parameters in rats after 14 days of subcutaneous injection of CCl_4 and oral administration of Aspirin

Group/ Treatment	AST (U/L)	ALT (U/L)	ALP (U/L)	TP (g/dl)	ALB (g/dl)
Group I negative control	70.67 ± 1.5	22.33 ± 2.31	82.00 ± 24.25	4.80 ± 0.71	3.37 ± 0.29
Group II(2 mL/kg olive oil)	74.00 ± 00	22.00 ± 1.73	116.00 ± 1.73	6.23 ± 0.12	3.77 ± 0.12
Group III (2 mL/kg CCl_4)	234.33 ± 7.02[a]	69.33 ± 2.52[a]	330.67 ± 17.04[a]	4.17 ± 0.12[a]	2.83 ± 0.25[a]
Group IV(1 mL/kg Aspirin)	189.33 ± 15.31[a]	52.33 ± 4.16[a]	204.33 ± 7.37[a]	4.37 ± 0.35[a]	3.00 ± 0.10[a]
$LSD_{0.05}$	11.62	18.00	26.33	0.34	0.09

Values are expressed as mean ± standard deviation of 3 replicates; a= significant difference at $p < 0.05$ when CCl_4-induced group or Aspirin-induced group is compared with Groups I and II; AST = Aspartate Aminotransferase; ALT = Alanine Aminotransferase; ALP = Alkaline Phosphatase; TP = Total Protein; ALB = Albumin.

Table 2. Serum levels of biochemical parameters in aspirin-treated rats after 14 days of oral administration of SS, NL, mixture of NL and AN extracts.

Group/ Treatment	AST (U/L)	ALT (U/L)	ALP (U/L)	TP (g/dl)	ALB (g/dl)
Group I negative control	70.67 ± 1.5	22.33 ± 2.31	82.00 ± 24.25	4.80 ± 0.71	3.37 ± 0.29
Group IV(1 mL/kg Aspirin)	189.33 ± 15.31[a]	52.33 ± 4.16[a]	204.33 ± 7.37[a]	4.37 ± 0.35[a]	3.00 ± 0.10[a]
Group VII (1 mL/kg Aspirin +1 mL/kg SS)	154.00 ± 11.36[a,b]	39.67 ± 2.52	149.33 ± 7.51[a,b]	5.20 ± 0.10[a,b]	3.33 ± 0.21[b]
Group VIII (1 mL/kg aspirin+2 mL/kg NL)	114.33 ± 6.66[a,b]	37.00 ± 2.00	124.67 ± 7.02[b]	5.47 ± 0.06[a,b]	3.20 ± 0.10[a,b]
Group X (1 mL/kg aspirin+ 2 mL/kg NL +1 mL/kg AN)	112.00 ± 0.00[a,b]	37.00 ± 0.00	120.00 ± 0.00[b]	5.80 ± 0.00[a,b]	3.30 ± 0.00[b]
$LSD_{0.05}$	11.62	18.00	26.33	0.34	0.09

Values are expressed as mean ± standard deviation of 3 replicates; a = significant difference at $p < 0.05$ when Aspirin-induced group or pretreated groups are compared with Group I; b = significant difference at $p < 0.05$ when pretreated groups are compared with Aspirin-induced group; AST = Aspartate Aminotransferase; ALT = Alanine Aminotransferase; ALP = Alkaline Phosphatase; TP = Total Protein; ALB = Albumin.

Table 3 reveals the results of mean serum levels/activities of AST, ALT, ALP, TP and ALB of the groups I, III, V, VI and IX. When the mean serum levels of AST, ALT, ALP, TP and ALB of group III are compared with controls (group I and II), the difference is statistically significant ($p < 0.05$). Mean serum levels of AST, ALT and ALP of the groups V, VI and IX were found to differ statistically ($p < 0.05$) being lower when compared with group III. In contrast, mean serum levels of TP and ALB of the groups V, VI and IX were found to differ statistically ($p < 0.05$) being higher than those of group III (CCl_4-induced group). Hence, the pre-treatment with SS, NL or a mixture of SS and CP induced significant reduction ($p < 0.05$) in the AST, ALT and ALP activities and at the same time brought significant elevation in the TP and ALB levels when compared with CCl_4-induced group (group III).

Table 3. Serum levels of biochemical parameters in CCl_4-treated rats after 14 days of oral administration of SS, NL, mixture of SS and CP extracts**.**

Group/ Treatment	AST (U/L)	ALT (U/L)	ALP (U/L)	TP (g/dl)	ALB (g/dl)
Group I negative control	70.67 ± 1.5	22.33 ± 2.31	82.00 ± 24.25	4.80 ± 0.71	3.37 ± 0.29
Group II (2 mL/kg olive oil)	74.00 ± 00	22.00 ± 1.73	116.00 ± 1.73	6.23 ± 0.12	3.77 ± 0.12
Group III (2ml/kg CCl_4)	234.33 ± 7.02[a]	69.33 ± 2.52[a]	330.67 ± 17.04[a]	4.17 ± 0.12[a]	2.83 ± 0.25[a]
Group V (2 mL/kg CCl_4 + 1 mL/kg SS)	155.67 ± 14.19[a,c]	50.00 ± 1.00[a,c]	242.33 ± 30.[a,c]	4.70 ± 0.10[c]	3.00 ± 0.10[a,c]
Group VI (2ml/kg CCl_4 + 2 mL/kg NL)	123.33 ± 5.03 [a,c]	42.00 ± 2.65[a,c]	180.00 ± 5.57[a,c]	5.07 ± 0.38[a,c]	3.17 ± 0.21[a,c]
Group IX (2ml/kg CCl_4+ 1 mL /kg SS+ 2 mL/kg CP)	76.33 ± 1.15 [c]	30.00 ± 1.73[c]	103.67 ±19.63[c]	6.03 ± 0.06[c]	3.50 ± 0.00[a,c]
$LSD_{0.05}$	11.62	18.00	26.33	0.34	0.09

Values are expressed as mean ± standard deviation of 3 replicates; a = significant difference at $p < 0.05$ when CCl_4-induced group or pretreated groups are compared with Groups I and II; c = significant difference at $p < 0.05$ when the pretreated groups are compared with CCl_4-induced group; AST = Aspartate Aminotransferase; ALT = Alanine Aminotransferase; ALP = Alkaline Phosphatase; TP = Total Protein; ALB = Albumin.

Table 4. Serum levels of biochemical parameters in CCl_4 aspirin-treated rats after 14 days of oral administration of SS, NL, mixture of SS and CP, and mixture of NL and AN **extracts.**

Group	Treatment	TB (mg/dl)	DB (mg/dl)
Group I	negative control	0.07 ± 0.02	0.04 ± 0.00
Group II	2 mL/kg olive oil	0.06 ± 0.00	0.05 ± 0.01
Group III	2 mL/kg CCl_4	0.11 ± 0.01	0.08 ± 0.01
Group IV	1 mL/kg aspirin	0.07 ± 0.02	0.07 ± 0.01
Group V	2 mL/kg CCl_4 + 1 mL/kg SS	0.06 ± 0.01	0.05 ± 0.01
Group VI	2 mL/kg CCl_4 + 2 mL/kg NL	0.06 ± 0.01	0.05 ± 0.01
Group VII	1 mL/kg aspirin + 1 mL/kg SS	0.06 ± 0.01	0.05 ± 0.01
Group VIII	1 mL/kg aspirin + 2 mL/kg NL	0.07 ± 0.00	0.05 ± 0.01
Group IX	2 mL/kg CCl_4 + 1 mL/kg SS + 2mL/kg CP	0.08 ± 0.01	0.06 ± 0.01
Group X	1 mL/kg aspirin + 2 mL/kg NL + 1 mL/kg AN	0.07 ± 0.00	0.06 ± 0.00

Values are expressed as mean ± standard deviation of 3 replicates; TB = Total Bilirubin; DB = Direct Bilirubin.

Table 4 reveals the results of mean serum levels/activities of TB and DB of the groups I to X. When mean serum levels of DB and TB of the groups III and IV are compared with controls (group I and group II), the difference is statistically insignificant ($p > 0.05$). Mean serum levels of DB and TB of the groups III and IV were found to be slightly ($p > 0.05$) being higher when compared with controls (group I and II) and the groups V, VI, VII, VIII, IX and X. Thus, the oral administration of SS, NL, a mixture of SS and CP, and a mixture of NL and

AN extracts caused slight reduction in the TB and DB activities when compared with the groups III and IV.

4. Discussions

Liver function tests are of immense importance in the diagnosis and monitoring of liver diseases. Serum levels of AST, ALT and ALP were assayed with a view to testing the liver injury and/or cholestasis (Burtis *et al.*, 2008; Vasudevan *et al.*, 2013). In the same vein, serum levels of TB and DB, and TP and ALB were investigated with the aim of testing hepatic excretory and synthetic functions respectively (Burtis *et al.*, 2008; Vasudevan *et al.*, 2013). The result of this study indicated that the subcutaneous injection of CCl_4 or the oral administration of aspirin induced a marked and significant elevation ($p < 0.05$) in ALT, AST and ALP activities, and conversely caused a significant reduction ($p < 0.05$) in TP and ALB levels (Table 1). These findings are corroborated with the findings of previous studies on CCl_4 and aspirin-induced hepatic damage by Kannan *et al.* (2013), Gupta *et al.* (2004), Adekeye *et al.* (2014), Mohammed *et al.* (2014), Adewoga and Sebiomo (2014) and Lee *et al.* (2011). The results in Table 2 revealed that the pre-treatment with SS, NL or a mixture of NL and AN extracts caused significant reduction ($p < 0.05$) in AST, ALT and ALP activities and conversely, induced significant elevation in TP and ALB levels. However, the co-administration with the NL and AN extract is by far efficacious in antagonizing the liver damage than do the SS or NL extracts alone. These findings were tallied with the findings of previous studies on aspirin-induced hepatic damage by Kannan *et al.* (2013), Gupta *et al.* (2004), Adewoga and Sebiomo (2014) and Lee *et al.* (2011). The significant reduction in the AST, ALT and ALP activities, and the induced elevation in the TP and ALB levels following the administration of the SS, NL or a mixture of NL and AN extracts may be attributed to the presence of phytochemicals found in SS, NL and AN as reported formerly by Kawanga and Bosch (2007), and Mebrahton *et al.* (2016), Madhusudhanan *et*

al. (2011), Afolayan *et al.* (2013), Amos *et al.* (1999) and Deshpande (2011). The results in Table 3 show that the pre-treatment with SS, NL or a mixture of the SS and CP extracts induced a marked and significant reduction ($p < 0.05$) in the AST, ALT and ALP activities and conversely caused a significant elevation in the TP and ALB levels; the co-administration of SS and CP extracts antagonized the liver damage in comparison with the control groups. The findings of the current study conformed to the findings of Usman *et al.* (2017), Adekeye *et al.* (2014), Adesanoye *et al.* (2010), Nafiu *et al.* (2011), Mohammed *et al*. (2014), Al-malki *et al*. (2013). The marked decrease in the AST, ALT and ALP activities, and the significant increase in the TP and ALB levels following the administration of SS, NL or a mixture of the SS and CP extracts may be attributed to the presence of phytochemicals found in SS, NL and CP as confirmed earlier by Kawanga and Bosch (2007), and Mebrahton *et al.* (2016), Madhusudhanan *et al.* (2011), Afolayan *et al.* (2013), Nafiu *et al.* (2011) and Isah *et al.* (2013). The results in Table 4 entailed that the oral administration of SS, NL, a mixture of SS and CP, and a mixture of NL and AN extracts caused slight reduction in the TB and DB activities when compared with both controls (group I and II) and positive controls (group III and IV). These findings are also corroborated with the findings of Adekeye *et al.* (2014) and Mohammed *et al*. (2014). Although the excretory function of the liver from this study was not significantly perturbed, the oral administration of SS, NL, a mixture of SS and CP, or a mixture of the NL and AN extracts was capable of antagonizing the liver damage caused by CCl_4 and aspirin in comparison with the control groups. This may be attributed to the presence of phytochemicals found in SS, NL , CP and AN as reported formerly by Kawanga and Bosch (2007), and Mebrahton *et al.* (2016), Madhusudhanan *et al.* (2011), Afolayan *et al.* (2013), Nafiu *et al.* (2011), Isah *et al*. (2013), Amos *et al.* (1999) and Deshpande (2011).

5. Conclusion

According to this study, liver damage at 16 mL CCl_4 dissolved in up to 100 mL olive oil and 9900 mg aspirin dissolved in up to 100 mL distilled water was apparent. Pretreatment of CCl_4-treated and aspirin-treated groups with SS, NL, a mixture of SS and CP or a mixture of NL and AN extracts orally administered at a dose of 250 mg/kg were efficacious in terms of antagonizing the liver damage caused by CCl_4 and aspirin.

Acknowledgement

The authors of this study acknowledge and thank the Department of Biological Sciences, Faculty of Science at Federal University, Kashere, Gombe State, Nigeria for their guidance during the course of writing this article.

Competing Interests

The authors have declared that no competing interests exist.

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JJBS

Jordan Journal of Biological Sciences

Volume 11, Number 4, September 2018
ISSN 1995-6673
Pages 389 - 393

Localities of Flower Chafers (Coleoptera: Scarabaeidae: Cetoniinae) in the Palestinian Territories (West Bank)

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Received January 11, 2018; Revised March 21, 2018; Accepted April 6, 2018

Abstract

Flower chafers (Cetoniinae) were collected from several habitats in the Palestinian Territories (West Bank) during several field trips conducted by the team at the Palestine Museum of Natural History. Nine species in four genera (*Aethiessa mesopotamica*, *Protaetia cuprea ignicollis*, *Protaetia funebris*, *Protaetia judith*, *Protaetia subpilosa*, *Oxythyrea cinctella*, *Oxythyrea noemi*, *Tropinota hirta suturalis*, and *Tropinota squalida pilosa*) were recorded. This study records additional localities of flower chafers in the Palestinian Territories.

Keywords: Flower chafers, Cetoniinae, Palestinian Territories, Distribution, Systematics.

1. Introduction

Scarabeidae are found all over the world except in Antarctica. There are over 30,000 described species, with the subfamily Cetoniinae being of more than 4000 described species (Counts and Hasiotis, 2009). Twenty nine species of flower chafers were recorded from the Levant (Katbeh-Bader and Barbero, 1999; Tauzin and Rittner, 2012). Several studies dealt with the Cetoniinae of the historic Palestine localities (Bodenheimer, 1935; 1937; Chikatunov and Pavliček, 1997; Chikatunov *et al*., 1999; Rittner and Sabatinelli, 2010; Sabatinelli *et al*., 2010; Tauzin and Rittner, 2012). Table 1 summarizes all the previously recorded species.

The insect fauna of the Palestinian territories are poorly studied and requires additional investigation (Qumsiyeh and Isaaq, 2012). The establishment of the Palestine Museum of Natural History (PMNH) makes it easy to focus on taxonomical studies, and to study the West Bank (Qumsiyeh *et al*., 2017).

This communication documents flower chafers' collection at the Palestine Museum of Natural History (PMNH) at Bethlehem University.

2. Materials and Methods

All specimens were collected from the Palestinian Territory of the West Bank by the Palestine Museum of Natural History team. A total of thirty-one areas were visited during trips from March, 2013 until June, 2017 (Table. 2). Flower chafers (Cetoniinae) were collected by a hand net on flowers from various localities and habitats, specimens were prepared and deposited at PMNH. Identification was based on Rittner and Sabatinelli (2010), Sabatinelli *et al.* (2010) and Tauzin and Rittner (2012).

3. Results

Totally, nine species of the subfamily Cetoniinae was found in the Palestinian territories (West Bank). These include: (*Aethiessa mesopotamica* Burmeister, 1842, *Protaetia cuprea ignicollis* (Gory and Percheron, 1833), *Protaetia funebris* (Gory and Percheron, 1833), *Protaetia judith* (Reiche, 1871), *Protaetia subpilosa* (Desbrochers des Loges, 1869), *Oxythyrea cinctella* (Schaum, 1841), *Oxythyrea noemi* Reiche and Saulcy, 1856, *Tropinota hirta suturalis* (Rritter, 1913), and *Tropinota squalida pilosa* (Brulle, 1832)). Table (2) lists all Cetoniini reported from historic Palestine. Rittner and Sabatinelli (2010) revised species of the genus *Oxythyrea*.

Aethiessa mesopotamica Burmeister, 1842 (Figure 1-C)

Materials Examined: Bardalla (PMNH4056, PMNH 4057, 18.4.2014); Jiftlik (PMNH1708-8, PMNH1708-9, PMNH1708-15, PMNH1708-16, 27.3.2013); Abu Dees (PMNH1728-2, 22.4.2013); Yatta (PMNH E10532, 8.4.2013; PMNH E10409, 25.3.2017).

Remarks: Tauzin and Rittner (2012) include records from Ramallah, Jenin and 60 km North of Jericho, collected during March and April from the Jordan Valley and Mediterranean habitats reaching the most southern parts of the West Bank (Figure 2). *Aethiessa mesopotamica* is an east Mediterranean species. Katbeh-Bader and Barbero, (1999) recorded it from Jordan.

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Table 1. List of Cetoniini reported from historic Palestine

Species	Localities	Reference
Aethiessa mesopotamica	Ramallah, Jenin, N Jericho	Bodenheimer (1937), Tauzin and Rittner (2012)
Aethiessa floralis	Palestine	Bodenheimer (1937)
Aethiessa inhumata	Palestine	Bodenheimer (1937)
Cetonia delagrangei	Ramallah	Tauzin & Rittner (2012)
Oxythyrea abigail	Gaza	Bodenheimer (1935; 1937), Tauzin and Rittner (2012)
Oxythyrea cinctella	Mikhmas, Massada, Jerusalem	Bodenheimer (1937), Rittner and Sabatinelli (2010), Tauzin and Rittner (2012)
Oxythyrea noemi	Ein Gedi, Jericho, Jerusalem, Mikhmas, Wadi Qilt.	Bodenheimer (1935; 1937) listed as *Oxythyrea funesta*, Chikatunov and Pavliček (1997) listed as *Oxythyrea funesta*,, Rittner and Sabatinelli (2010), Tauzin and Rittner (2012)
Protaetia (*Cetonischema) speciosa jousselini*	Jerusalem	Tauzin and Rittner (2012)
Protaetia (Potosia) angustata	Palestine	Bodenheimer (1937) as *Potosia angustata*
Protaetia (*Eupotosia*) *mirifica koenigi*	Jerusalem	Tauzin & Rittner (2012)
Protaetia (*Eupotosia*) *affinis pyrodera*	Jerusalem	Bodenheimer (1937) as *Potosia affinis*, Tauzin and Rittner (2012)
Protaetia (*Netocia*) *afflicta*	Bethlehem, Gaza	Bodenheimer (1935) as *Netocia afflicta*, Bodenhiemer (1937) as *Potosia afflicta*, Tauzin and Rittner (2012)
Protaetia (*Netocia*) *subpilosa dorchini*	Bethlehem, Ramallah, Nablis	Bodenheimer (1937) as *Potosia subpilosa*, Tauzin and Rittner (2012)
Protaetia (*Netocia*) *trojana galathea*	Jerusalem	Bodenheimer (1937) as *Potosia sibirica*, Tauzin and Rittner (2012)
Protaetia (*Netocia*) *vidua*	Jerusalem	Bodenheimer (1937) as *Potosia vidua*, Tauzin and Rittner (2012)
Protaetia (*Potosia*) *cuprea*	Jerusalem	Bodenheimer (1935; 1937) as *Potosia cuprea ignicollis* Tauzin and Rittner (2012)
Protaetia (Potosia) funebris funesta	Jerusalem, Nablis	Bodenheimer (1937) as *Potosia funesta*, Chikatunov and Pavliček (1997), Chikatunov *et al.* (1999), Alpansèque and Tauzin (2006)
Protaetia (*Foveopotosia*) *judith*	Jerusalem	Bodenheimer (1937) as *Potosia judith*, Tauzin and Rittner (2012)
Stalagmosoma albellum	Palestine	Bodenheimer (1937) as *Stalagmopygus allbella*
Tropinota (s. str.) squalida pilosa	Ein Feshka, Jericho, Jerusalem, Qumran	Bodenheimer (1937) as *Epicometis squalida*, Bodenheimer (1937) as *Tropinota squalida*, Tauzin and Rittner (2012)
Tropinota (s. str.) vittula	Jerusalem	Bodenheimer (1937), Tauzin and Rittner (2012)
Tropinota (*Epicometis*) *hirta suturalis*	Jerusalem, Janin, Nablis Bethlehem, Ramallah	Bodenheimer (1937) as *Epicometis hirta*, Tauzin and Rittner (2012)

Table 2. List of visited localities and their coordinates.

Location	N	E	Location	N	E
Abu Dis	31° 45' 23.5188"	35° 15' 49.665"	Nabi Saleh	32° 0' 58.5792"	35° 7' 18.9048"
Ain Samia	31° 58' 35.925"	35° 20' 29.9184"	Nahaleen	31° 40' 54.336"	35° 7' 9.0156"
Al Ogga	31° 57' 6.2418"	35° 28' 33.1788"	Rtas	31° 41' 18.9414"	35° 11' 14.9706"
Bardalla	32° 23' 12.6816"	35° 28' 40.9038"	Silit Al Daher	32° 18' 59.835"	35° 11' 17.4438"
Beit Jaad	32° 28' 12.0864"	35° 21' 24.6096"	Slafit	32° 4' 39.7092"	35° 11' 14.9706"
Beit Ta'mar	31° 40' 19.0992"	35° 16' 37.5594"	Taibe	31° 57' 31.1472"	35° 17' 54.8052"
Beni Nuaim	31° 30' 44.4738"	35° 9' 51.699"	Tarqumia	31° 34' 37.5234"	35° 1' 36.8502"
Bethlehem	31° 43' 4.242"	35° 12' 20.9412"	Wadi Al Makhrour	31° 42' 58.0968"	35° 9' 27.4428"
Bir Zait	31° 57' 31.4382"	35° 11' 3.8466"	Wadi Fukeen	31° 42' 15.7746"	35° 6' 5.0544"
Dayr Ballout	32° 3' 26.0496"	35° 1' 59.4084"	Wadi Haramya	31° 59' 55.8558"	35° 13' 55.6458"
Jenin	32° 28' 35.1588"	35° 17' 19.428"	Wadi Nar	31° 43' 44.8998"	35° 17' 8.5344"
Jiftlik	32° 8' 33.0822"	35° 29' 54.135"	Wadi Qana	32° 9' 26.6142"	35° 7' 2.2188"
Kufr Al Deek	32° 3' 35.3442"	35° 5' 1.8672"	Yatta	31° 25' 58.1982"	35° 6' 52.9488"
Mar Saba	31° 42' 15.7278"	35° 19' 52.5324"	Ze'im	31° 47' 11.1222"	35° 15' 53.373"
Mikhmas	31° 51' 52.7934"	35° 16' 12.3744"			

Protaetia* (*Potosia*) *cuprea ignicollis (Gory et Percheron, 1833) (Figure 1-B)

Materials Examined: Wadi Al Quff (PMNH4535, PMNH 4536, 3.5.2014); Nabi Saleh (PMNH1736-23, PMNH1736-24, 3.5.2013); Kufr Zabad (PMNH1755-9, PMNH1755-10, PMNH1755-12, 18.5.2013); Dayr Ballout (PMNH E10618, 28.4.2017). observed in Bethlehem.

Remarks: This is one of the common species of flower chafers in the West Bank (Figure 2). It is common in the Levant, and can be found in the eastern and southern Mediterranean (Chikatunov *et al.,* 1999). It exhibits color variation from shiny green to green red and blue green, while green and red green are the dominant colors of specimens collected in the West Bank (Tauzin and Rittner, 2012)

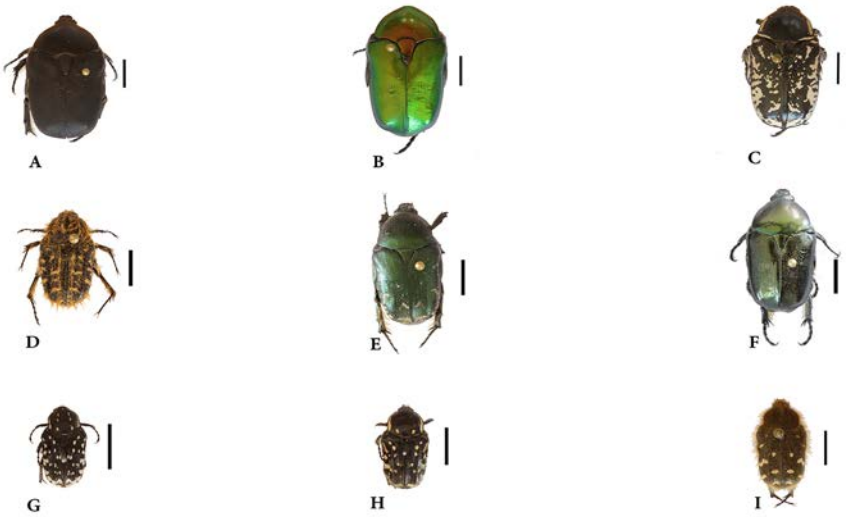

Figure 1. A: *Protaetia funebris*. B. *Protaetia cuprea*. C: *Aethiessa mesopotamica*. D. *Tropinota squalida pilosa*. E. *Protaetia subpilosa*. F. *Protaetia (Foveopotosia) judith*. G. *Oxythyrea noemi*. H: *Oxythyrea cinctella*. I. *Tropinota hirta suturalis*. bar = 5mm.

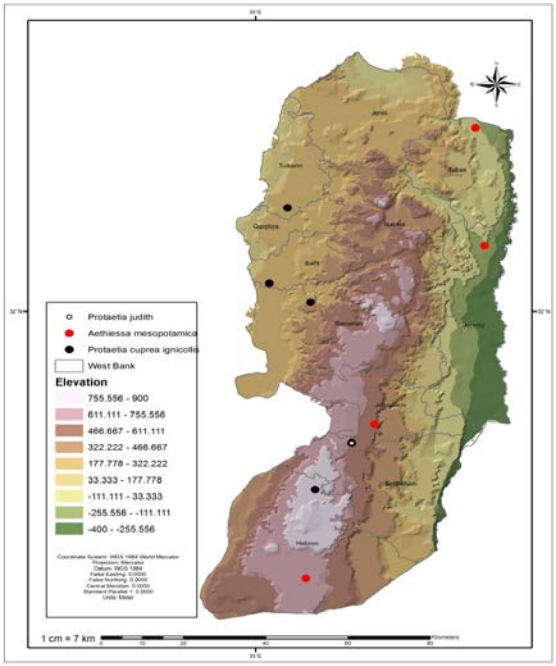

Figure 2. Distribution of *Aethiessa mesopotamica* (Red circles), *Protaetia cuprea ignicollis* (Black circles) and *Protaetia* (*Foveopotosia*) *judith* (open circle) in the Palestinian Territories.

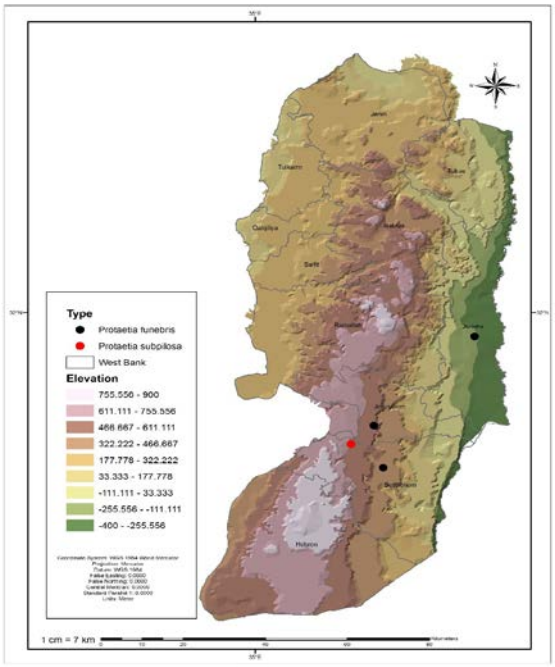

Figure 3. Distribution of *Protaetia subpilosa* (Red circle) and *Protaetia funebris* (Black circle) in the Palestinian Territories**.**

Protaetia (Netocia) subpilosa (Desbrochers des Loges, 1869) (Figure 1-E)

Materials Examined: Bethlehem (PMNH6434, 1.5.2015).

Remarks: A single specimen was collected from Bethlehem (Figure 3). This is a small species of flower chafers. Its distribution extends along the East-Mediterranean including Greece, Turkey, Cyprus, Syria, Lebanon, Jordan and Palestine (Chikatunov *et al.,* 1999). *P. subpilosa* varies in color from green, green blue and in rare situation black color (Tauzin and Rittner, 2012).

Protaetia (Potosia) funebris (Gory and Percheron, 1833) (Figure 1-A)

Materials Examined: Abu Dees (PMNH1728-1, 22.4.2013); Beit Ta'mar (PMNH5284, 22.10.2014); Al Ogga (PMNH5977, 9.3.2015).

Remarks: *Protaetia funebris* is the largest species of Flower Chafers collected from the West Bank. Figure (3) shows the distribution of this species. This species is known in the Levant (Chikatunov *et al.,* 1999). Alpansèque and Tauzin, (2006) shows the complexity of this species and the colour variation.

Protaetia* (*Foveopotosia*) *judith (Reiche, 1871) (Figure 1-F)

Materials Examined: Bethlehem (PMNH E10689, 24.11.2017)

Remarks: This is a rare species in the West Bank (Figure 2). It was recorded from several localities in northern Palestine (Tauzin and Rittner, 2012), with records around Jerusalem. Tauzin and Rittner (2012) stated that this species is limited to the Eastern edge of the Mediterranean (Rhodos, Cyprus, Turkey Syria and Lebanon).

Tropinota hirta suturalis (Rritter, 1913) (Figure 1-I)

Materials Examined: Bir Zait (PMNH3972, 15.4.2014); Bethlehem (PMNH5195, 21.3.2014); Al Ogga (PMNH5867, 9.3.2015); Salfit (PMNH6834, April. 2015 ; PMNH6838, March. 2015); Wadi Al Makhrour (PMNH7594, 23.9.2015); Wadi Fukeen (PMNH7602, PMNH7611, PMNH7613, 7.7.2016); Wadi Al Haramya (PMNH7684, 3.3.2016); Kufr Al Deek (PMNH E10569 & E10588-92, March.2017).

Remarks: This is a common species always found with *Oxythyrea noemi* on different species of flowers. Figure (4) shows the distribution of this species. This species is known from Europe and the Middle East (Chikatunov *et al.*, 1999; Tauzin and Rittner, 2012). *Tropinota hirta suturalis* is the only subspecies that is found in the Euro-Mediterranean area (Tauzin and Rittner, 2012).

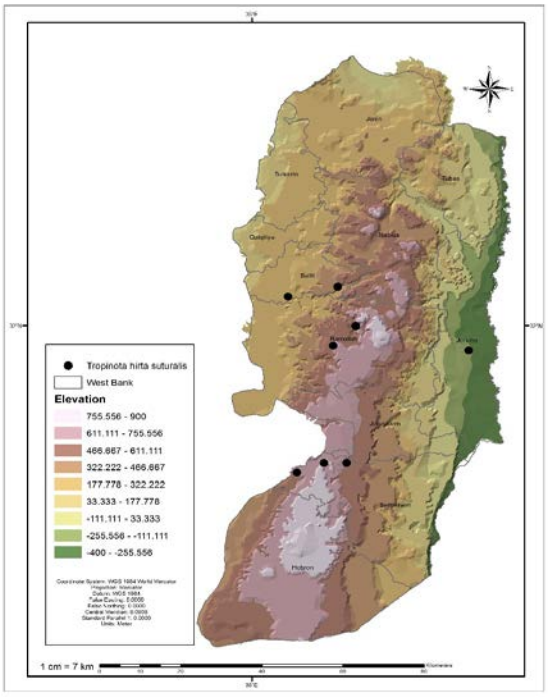

Figure 4. Distribution of *Tropinota hirta suturalis* in the Palestinian Territories**.**

Tropinota squalida pilosa (Brulle, 1832) (Figure 1-D)

Materials Examined: Wadi Al Quff (PMNH3721, PMNH3722, 28.2.2014); Mar Saba (PMNH4068, 13.1.2014); Al Ogga (PMNH1710-13, 27.3.2013; PMNH5870, 9.3.2015).

Remarks: This subspecies is known from North Africa to the Levant (Chikatunov *et al.*, 1999; Tauzin and Rittner, 2012). Figure 5 shows its distribution in the West Bank. Sabatinelli *et al*. (2010) described the morphological differences between *T. squalida* and *T. vittula*, supporting the idea that shows that *T. vittula* is a separate species and not a subspecies.

Oxythyrea cinctella (Schaum, 1841) (Figure 1-H)

Materials Examined: Bethlehem (PMNH6092, 2.4.2015); Jiftlik (PMNH1708-3, 27.3.2013); Rtas (PMNH1711-12, 31.3.2013); Bani Nua'im (PMNH1714-20, 7.4.2013); Abu Dees (PMNH1728-4, 22.4.2013).

Remarks: This species is distributed in south Eastern Europe extending from Turkey and the southern states of the former Soviet Union and as far as China (Smetana, 2006). Usually *O. cinctella* is found with *Oxythyrea noemi* (Rittner and Sabatinelli, 2010). Figure 5 shows its distribution in the West Bank. In this study, specimens were collected during early spring (March and April).

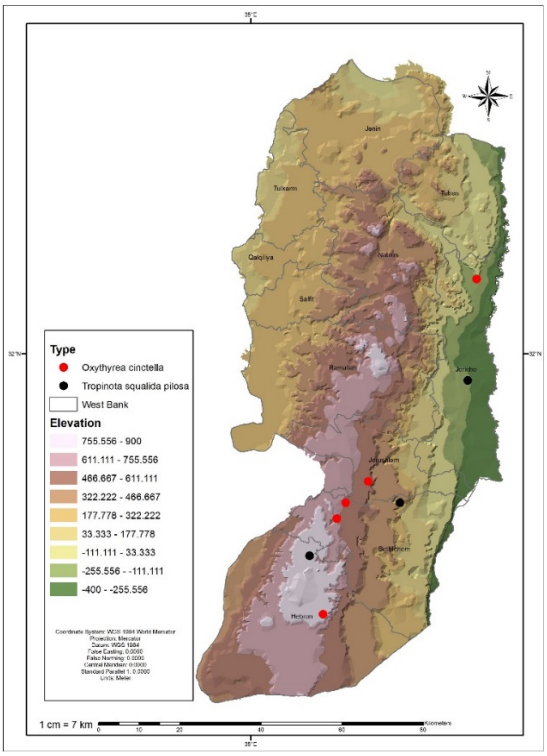

Figure 5. Distribution of *Oxythyrea cinctella* (Red circles) and *Tropinota squalida pilosa* (Black circles) in the Palestinian Territories**.**

Oxythyrea noemi Reiche and Saulcy, 1856 (Figure 1-G)

Materials Examined: Wadi Al Quff (PMNH3937, 22.3.2014 ; PMNH4344, 3.5.2014); Al Ogga (PMNH5972, 9.3.2015); Bethlehem (PMNH6085, PMNH 6091, PMNH6095, 2.4.2015 ; PMNH7689, 6.3.2016); Al Makhrour (PMNH6169, 15.4.2015); Beit Jaad (PMNH 6607, 19.3.2015); Wadi Fukeen (PMNH7612, PMNH 7615, 7.7.2016); Jenin (PMNH7781, PMNH7783, April.2016 ; PMNH7833, PMNH7834, PMNH7840, 12.4.2016); Jiftlik (PMNH1708-1, PMNH1708-4, PMNH 1708-6, 27.3.2013); Rtas (PMNH1711-7, 31.3.2013); Tarqumia (PMNH1712-1, PMNH1712-10, PMNH1712-13, PMNH1712-19, PMNH 1712-6, PMNH 1712-9, 4.4.2013); Wadi Nar (PMNH1727-3, 13.4.2013); Ain Samia (PMNH1731-4, PMNH1731-7, 12.4.2013); Mikhmas (PMNH1733-3, 1733-4, 27.4.2013); Taibe (PMNH1734-11, 12.4.2013); Nahaleen (PMNH1735-11, 1735-12, 1735-13, 2.5.2013); Nabi Saleh (PMNH1736-16,

PMNH 1736-19, PMNH1736-27, PMNH1736-29, 3.5.2013); Ze'im (PMNH1749-11, PMNH1749-13, PMNH 1749-4, 13.5.2013); Kufr Zabad (PMNH1755-10, 18.5.2013); Wadi Qana (PMNH1756-28, 17.5.2013); Silit Al Daher (PMNH 1809-6, 14.6.2013); Yatta (PMNH E10369, 25.3.2017); Kufr Al Deek (PMNH E10587, PMNH E10586, March. 2017); Dayr Ballout (PMNH E10672, PMNH E10672, April. 2017).

Remarks: This is the most common species found in the West Bank, inhabiting various types of habitats (Figure 6). It was reported from across the Levant, Egypt and Cyprus (Rittner and Sabatinelli, 2010).

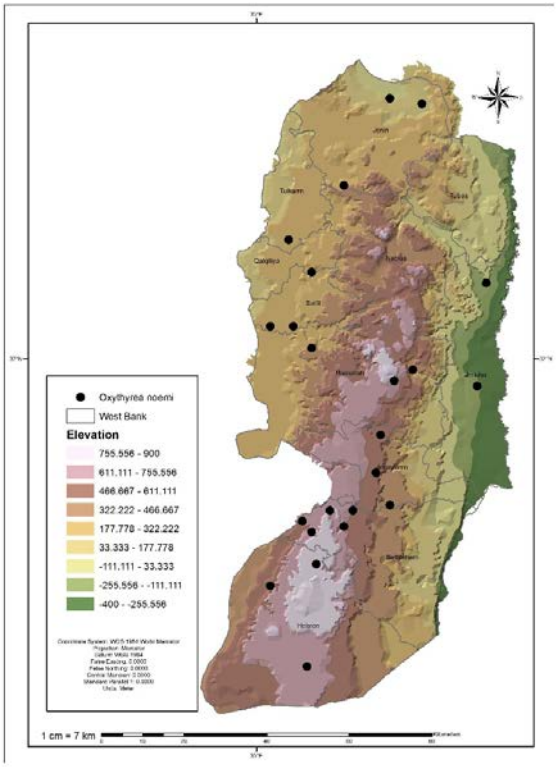

Figure 6. Distribution of *Oxythyrea noemi* (Black circles) in the Palestinian Territories**.**

4. Discussion

Within the Levant, Tauzin and Rittner (2012) listed twenty-nine species. So far, a total of twenty-two species of Cetoniinae have been reported from Palestine (see table 1). According to Tauzin and Rittner (2012), the distribution patterns of the Cetoniinae of Palestine shows several affinities. Of the reported species in this study, *Oxythyrea noemi*, *Protaetia afflicta*, *Protaetia judith*, *Protaetia affinis* are considered eastern Mediterranean, *Aethiessa mesopotamica* and *Protaetia funebris* are southwestern Asiatic, *Tropinota squalida* is Mediterranean, *Tropinota hirta* spp. is European-Mediterranean, while *Oxythyrea cinctella* is Turano-Mediterranean.

Rittner and Sabatinelli (2010) re-examined specimens of *Oxythyrea funesta* at Tal Abib Museum, and found that these specimens are actually *Oxythyrea noemi*, and considered all previous records of *O. funesta* listed in Bodenheimer (1937) and Chikatunov and Pavliček (1997) are *O. noemi*.

Further studies should be conducted over longer periods and cover different habitats in the Palestinian territories to update the distribution of this little known group.

Acknowledgment

We are grateful for the field support of Mubarak Zawahra, Mohammad Najajreh, and Khawla Abualia and Prof. Dr. Mazin Qumsiyeh. We thank Eva Amar for the maps' preparation.

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JJBS

Jordan Journal of Biological Sciences

Volume 11, Number 4, September 2018
ISSN 1995-6673
Pages 395 - 399

Alterations in the Serum Electrolytes of the Indian Skipper Frog *Euphlyctis cyanophlyctis* caused by an Organophosphate Pesticide: Chlorpyrifos

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Received January 5, 2017; Revised February 11, 2018; Accepted March 2, 2018

Abstract

The aim of the present study is to determine the changes in blood electrolytes (calcium and phosphate) in the Indian skipper frog *Euphlyctis cyanophlyctis* following short-term and long-term treatments with chlorpyrifos. To determine the effects of short-term exposure, the frogs were exposed to 3.99 mg/L of chlorpyrifos (i.e. 0.8 of 96 h LC_{50} value) for ninety-six hours. To investigate the effects of long-term exposure, the frogs were exposed to 0.99 mg/L (0.2 of 96 h LC_{50} value) of chlorpyrifos for 30 days. The frogs were sacrificed after 24, 48, 72 and 96 hours (for short-term experiment) or after 5, 10, 15 and 30 days (long-term experiment). Blood samples were collected and serum calcium and phosphate levels were analyzed. Student's t test was used to determine the statistical significance difference between the experimental group and its specific-time control group. Exposure of the frog *Euphlyctis cyanophlyctis* to chlorpyrifos causes a decrease in the serum calcium levels after 48 hours. This decrease continued up to the end of the experiment (96 hours). The serum inorganic phosphate levels decrease progressively 72 hours onwards following the chlorpyrifos exposure. In the long-term experiment, the first perceivable change has been noticed on day ten in the serum calcium as the levels decreased at this interval. The levels continued to fall progressively till the end of the experiment (thirty days). The serum phosphate levels of the chlorpyrifos-treated *Euphlyctis cyanophlyctis* show a decrease on day ten and fifteen. However, on day thirty, the levels were almost normal. The changes noticed in the blood electrolytes may cause disturbances in the vital physiological functions of the frog, growth and even its ability to survive in nature.

Keywords: Amphibia, chlorpyrifos, organophosphate, serum calcium, serum phosphate, *Euphlyctis cyanophlyctis*

1. Introduction

Organophosphorus pesticides are widely used around the world although they lack target specificity, and have severe effects on aquatic non-target animals (Fulton and Key, 2001; Yan *et al*., 2008). Chlorpyrifos, a non-systemic organophosphate pesticide, is one of the most widely used insecticides on a variety of crops and in numerous non-agricultural situations (WHO, 2009). Amphibians are sensitive to most pesticides when exposed through direct overspray, pesticide drift, rainfall and run-off into water bodies. Many amphibians breed within or near agricultural areas that are usually exposed to pesticides (Palenske *et al.*, 2010), thus both the larvae and adults are exposed to pesticides at all life stages, either in the waters (larvae) or on land (adults). This can lead to a decline in their global population which is a major concern now-a-days (Sparling, 2003; Relyea, 2005; Hayes *et al.*, 2006; Mccallum, 2007; Todd *et al.*, 2011; Whittaker *et al*., 2013; Arntzen *et al.*, 2017; Srivastav *et al.*, 2016, 2017).

Chlorpyrifos is highly toxic to amphibians (Davidson *et al.*, 2012). Residues of chlorpyrifos have been found in the Pacific tree frog tadpoles (Datta *et al*., 1998). Jayawardena *et al.* (2011) have noticed profound effects in amphibians after a chronic exposure to chlorpyrifos. Bernabo *et al.* (2011) exposed frog tadpoles to chlorpyrifos and noticed that 20-25 % of the exposed tadpoles became intersex. The exposure to Chlorpyrifos in amphibians resulted in (i) damage to muscles (Colombo *et al*., 2005), (ii) reduced swim speed and activity in tadpoles (Wijesinghe *et al.*, 2011), (iii) reduced body length and mass (Richards and Kendall, 2003), and (iv) increased induction of micronuclei and chromosomal lesions in the erythrocytes (Yin *et al*., 2009).

Agrochemical contaminants, organophosphates and organochlorine pesticides have been reported to cause inhibition of AChE and malformations in frogs (Fort and

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Paul, 2002; Fort *et al*., 2004 a,b; Krishnamurthy and Smith, 2010; Hegde and Krishnamurthy, 2014). Palenske *et al*. (2010) have suggested that physiological studies provide a better understanding regarding the toxic effects of contaminants to aquatic organisms. Although several studies have been performed dealing with the effects of toxicants on amphibians, there exists no information regarding the effects of toxicants on amphibian calcium regulation. Calcium is vital for living organisms, and has been implicated in controlling a wide variety of physiological and biological functions. It seems very difficult to mention a physiological process that does not, in one way or another, depend on calcium. Hence, the present study aim to investigate the effects of chlorpyrifos on blood calcium and phosphate levels of the anuran, Indian skipper frog *Euphlyctis cyanophlyctis.*

2. Materials and Methods

Laboratory reared Indian skipper frogs, *Euphlyctis cyanophlyctis* (both sexes; body wt. 12-17 g) were selected and acclimatized for fifteen days in 30 L all glass aquaria. The frogs were not fed for twenty-four hours before and during the experiment. Short-term and long-term experiments have been performed. This study evaluates the possible effects of chlorpyrifos after acute exposure, i.e. short-term exposure to high doses of chlorpyrifos. The real exposure effects come after a long-term exposure using low-doses of chlorpyrifos which may result in varied effects compared to the acute exposure to high-doses of chlorpyrifos. This could be very useful in understanding the long-term effects of chlorpyrifos with low concentrations and comparing them with the short-term effects of chlorpyrifos at high concentrations.

(i) *Short-term Exposure***:** In this experiment, the frogs (n =24) were subjected to 0.8 of 96 h LC_{50} (LC_{50} value of chlorpyrifos described earlier by Srivastav *et al*., 2017) value of chlorpyrifos (3.99 mg/L) for ninety-six hours. Simultaneously, a control group (n =24) was also used for comparison. The frogs were kept in groups of ten each in 30 L media. Six frogs of the control and experimental groups were killed on each time intervals after a period of 24, 48, 72 and 96 hours of exposure.

(ii) *Long-term Exposure***:** The frogs (n =24) were exposed to 0.99 mg/L (0.2 of 96 h LC_{50} value) of chlorpyrifos for thirty days. Simultaneously, a control group (n =24) was also used for comparison. Six frogs from the control and experimental groups were sacrificed after 5, 10, 15 and 30 days of the toxicant treatment.

In each experiment, the frogs were slightly anesthetized with ether, and their blood samples were collected by cardiac puncture. The collected blood samples were allowed to clot at room temperature. Sera were separated by centrifugation (at 3000 rpm) and were kept at -20C until analysis for serum electrolytes using commercial diagnostic kits - calcium (calcium kit, Sigma-Aldrich) and inorganic phosphate (Pointe Scientific, USA). All determinations were carried out in duplicates for each sample. Animal handling and sacrifice were carried out in accordance with the guidelines provided by the Ethics Committee of the University (F.Sc.2551/Zoology/4-12-06).

All data were presented as the mean ± S.E. of six specimens, and the Student's t test was used to determine statistical significance. In all studies, the experimental group was compared to its specific-time control group.

3. Results

Short-term exposure of the frog *Euphlyctis cyanophlyctis* to chlorpyrifos results in a decrease in the serum calcium levels after forty-eight hours This decrease continued till the end of the experiment (96 h) (Figure 1). The serum inorganic phosphate levels remain unaffected till forty-eight hours following the chlorpyrifos exposure. The levels decreased progressively seventy-two hours onwards (Figure 2).

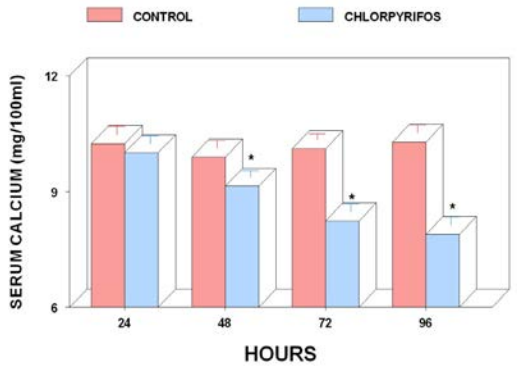

Figure 1. Serum calcium levels of short-term chlorpyrifos-treated *Euphlyctis cyanophlyctis*. Values are mean ± S.E. of six specimens. Asterisk indicates significant differences ($P< 0.05$) from control.

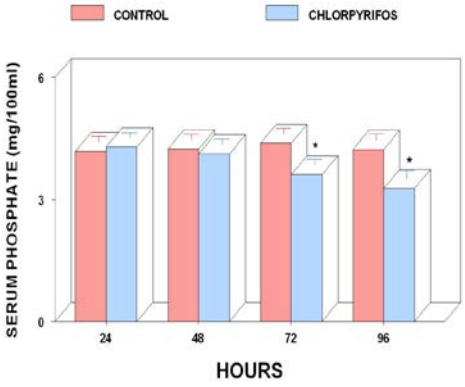

Figure 2. Serum phosphate levels of short-term chlorpyrifos-treated *Euphlyctis cyanophlyctis*. Values are mean ± S.E. of six specimens. Asterisk indicates significant differences ($P< 0.05$) from control.

In the long-term exposure of the *Euphlyctis cyanophlyctis* to chlorpyrifos, the first perceivable change has been noticed in the serum calcium by day ten; the levels decreased at this interval. The levels continued to fall progressively up to the end of the experiment (30 days; Figure 3). The serum phosphate levels of the chlorpyrifos-treated *Euphlyctis cyanophlyctis* showed a decrease on

days ten and fifteen. However, on day thirty, the levels were almost normal (Figure 4).

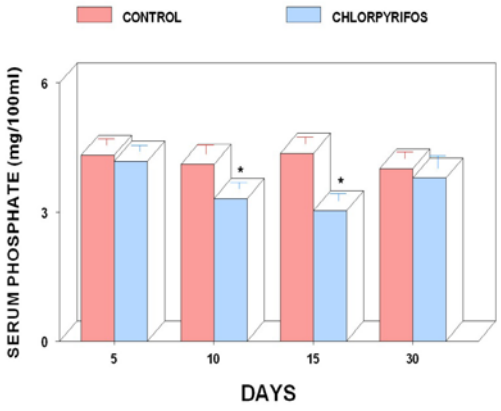

Figure 3. Serum calcium levels of long-term chlorpyrifos-treated *Euphlyctis cyanophlyctis*. Values are mean ± S.E. of six specimens. Asterisk indicates significant differences ($P< 0.05$) from control.

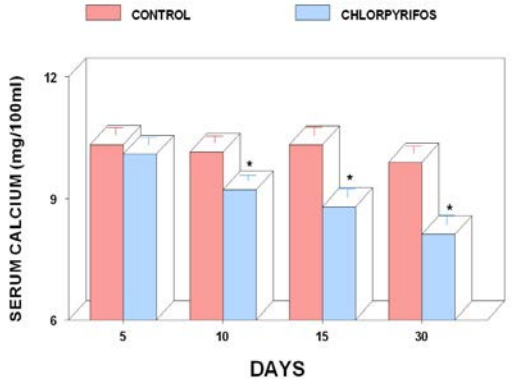

Figure 4. Serum phosphate levels of long-term chlorpyrifos-treated *Euphlyctis cyanophlyctis*. Values are mean ± S.E. of six specimens. Asterisk indicates significant differences ($P< 0.05$) from control.

4. Discussion

Chlorpyrifos exposure provoked hypocalcemia and hypophosphatemia in *Euphlyctis cyanophlyctis.* This study presents the first report regarding the effects of toxicants on the blood electrolytes of amphibians; No report preceding this study which tackles this issue has been found. The present study derives support from the reports of earlier workers who have also noticed hypocalcemia in other vertebrates after the exposure to chlorpyrifos (fish – Srivastav *et al.,* 1997 a; rats – Tripathi *et al.,* 2013), deltamethrin (fish – Srivastav *et al.,* 1997 b, 2010), cypermethrin (fish – Mishra *et al.,* 2011), lead (fish – Rai *et al.,* 2010, 2013), botanical pesticides (fish – Kumar *et al.,* 2011 a, b; Prasad *et al.,* 2011,2013) and cadmium (fish – Larsson *et al.,* 1981; Pratap *et al.,* 1989; Rai and Srivastav, 2003; Rai *et al.,* 2009; rabbits – Kenny, 1966; rats – Tripathi and Srivastav, 2011). Contrary to these reports, few studies have noticed either no effect (Oner *et al.,* 2008; Velisek *et al.,* 2009) or hypercalcemia (Sharma *et al*., 1982; Suzuki *et al*., 2006) after the exposure of fish to toxicants.

In the present study, hypophosphatemia has been noticed in the chlorpyrifos- treated *Euphlyctis cyanophlyctis.* This is in conformity with the reports of other investigators who have also noticed similar effects after the exposure of various fish and other species to toxicants (chlorpyrifos – Srivastav *et al.,* 1997 a; cadmium – Rai and Srivastav, 2003; deltamethrin – Srivastav *et al*., 1997 b; azadirachtin – Kumar *et al*., 2011 a; *Euphorbia tirucalli* – Kumar *et al.,* 2011 b; *Nerium indicum* – Prasad *et al.,* 2013; *Euphorbia royleana* – Prasad *et al.,* 2011); chicken (gammabenzene hexachloride and quinolphos –Agarwal *et al.,* 2009) and rats (cadmium – Tripathi and Srivastav, 2011; chlorpyrifos – Tripathi *et al*., 2013). In the present study, the serum phosphate levels in the frogs after a thirty-day chlorpyrifos-exposure increased approaching the control values. This could be explained as a redistribution of phosphate between the extracellular fluid and intracellular fluid.

Few researchers have noticed degeneration in kidney tubules after the treatment of amphibians with the toxicant (Hanafy and Soltan, 2007), fish (Srivastava *et al*., 1990; Akram *et al*., 1999) and mammals (Chmielnicka *et al*., 1989; Prozialeck *et al*., 2009; Tripathi and Srivastav, 2010). Mahmood *et al.* (2016) have reported increased metal concentrations in *Euphlyctis cyanophlyctis*, and also noticed degeneration in the kidney cells. The observed hypocalcemia and hypophosphatemia in the chlorpyrifos-treated *Euphlyctis cyanophlyctis* could be attributed to the kidney damage. It has been suggested that toxicant-induced renal lesions may cause hyperfiltration in the kidneys thus causing increased efflux of the electrolytes (Chmielnicka *et al.,* 1989; Prozialeck *et al.,* 2009). Schutte *et al. (*2008) have noticed increased calciuria in cadmium-exposed women. In the past, it has been suggested that renal tubule damage might be one of the main reasons for provoking hypocalcemia/hypophosphatemia in toxicant-exposed animals (Koyama and Itazawa, 1977; Roch and Maly, 1979; Larsson *et al*., 1981; Haux and Larsson, 1984; Rai and Srivastav, 2003; Srivastav *et al.,* 1997 a, b; Kumar *et al*., 2011 a, b; Prasad *et al*., 2011, 2013). Moreover, Patel *et al.* (2006) have also suggested that lead-induced ionoregulatory toxicity in rainbow trout is not exclusively a branchial phenomenon, but is in part a result of disturbances in the ionoregulatory mechanism of the kidneys.

In conclusion, the present study has revealed the consequences of the exposure to chlorpyrifos on alterations in the vital electrolytes of the frog *Euphlyctis cyanophlyctis.* The physiological capabilities of chlorpyrifos raise severe concerns regarding its danger to aquatic organisms. Further studies are needed to explore the biological consequences after the exposure of frogs to chlorpyrifos, and to formulate future strategies for encountering the amphibian population decline.

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JJBS

Volume 11, Number 4, September 2018
ISSN 1995-6673
Pages 401 - 405

Jordan Journal of Biological Sciences

Mycosynthesis of Silver Nanoparticles using *Terminia* sp. Desert Truffle, Pezizaceae, and their Antibacterial Activity

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Received February 8, 2017; Revised February 23, 2018; Accepted March 2, 2018

Abstract

Desert truffle *Tirmania* sp. is appropriate as a greener reducing agent to synthesize silver nanoparticles at room temperature in a dark place by utilizing the aqueous extract of ascocarps of *Tirmania* sp. and $10^{-3}AgNO_3$ solution. Characterization of those nanoparticles was accomplished by changing color, UV-visible spectrum, FT-IR, AFM and SEM analyses. The changing in color is considered a first indicator for the formation of AgNPs which changed from pale yellow to dark brown after seventy-two hours. UV-visible spectrum showed the formation of AgNPs at peak 415 nm. AFM and SEM images demonstrated nanoparticles with spherical or irregular-shaped, agglomerated and highly heterogeneous. The higher zone of inhibition was 14 mm against *Pseudomonas aeruginosa* at a concentration of 5 mg/well while, the lower zone of inhibition was 9.5 mm toward *Staphylococcus aureus* at the level of 5 mg/well. These *Tirmania*-AgNPs had a promising antibacterial activity toward gram-negative and gram-positive bacteria, mainly the infectious bacteria of the eyes, *Pseudomonas aeruginosa*.

Keywords: Ascocarp, Tuber, Antibacterial effects, Green nanotechnology, Fungi.

1. Introduction

Desert truffles are hypogeous ascomycetes which develop underground such as *Terfezia* sp. and *Tirmania* sp. which grow in mycorrhizal association with *Helianthemum* sp., Cistaceae family (Bradai *et al.*, 2014). Desert truffles grow naturally after rainfall in arid and semi-arid zones of some deserts in Middle Eastern and Arabian countries like Iraq, Saudi Arabia, Jordan, Syria, Turkey, Iran, Kuwait, United Arab Emirates, Qatar, Bahrain, Morocco, Algeria, Tunisia and Libya (Owaid, 2018). The desert of Iraq in Anbar province is rich in desert truffles especially *Terfezia* sp. and *Tirmania* sp. (Owaid, 2016).

These truffles have a sufficient nutritional value because of their compositions of proteins, crude fibers, polysaccharides, fat and low energy (Dogan and Aydin, 2013). *Tirmania* sp. is rich in fatty acids (Bokhary *et al*., 1989). Thus it is considered one of the natural health products (Dogan *et al.*, 2013). Truffles have therapeutic benefits including anticholinesterase, antioxidant (Tel-Cayan *et al*., 2018), anticancer, anti-cholesterol, anti-cardiovascular activities. They can be useful in improving the immune system, in the prevention of sleep and prostate disorders, hormonal imbalances in females and in increasing the absorption of calcium from milk (Gajos *et al*., 2014).

Several studies have discussed the antimicrobial effects of desert truffles especially against microbes (Owaid, 2018), such as *Pseudomonas aeruginosa* and *Staphylococcus aureus* (Janakat *et al*., 2004; Janakat *et al*., 2005). But only few researchers have investigated the biosynthesis of desert truffle-nanoparticles and their bioactivity recently. Muhsin and Hachim (2016) managed to synthesize AgNPs from *Tirmania nivea*, while Khadri *et al.* (2017) produced silver nanoparticles from *Terfezia claveryi* which showed excellent cytotoxicity toward the breast cancer cell line. Some researchers have focused on using macro-fungi as natural products to mycosynthesize metallic nanoparticles in the last decade. The increase in using edible mushrooms in the field of nanoscience can be attributed to the ability to produce huge amounts of fungal biomass (Owaid and Ibraheem, 2017).

This study is one of its kind conducted to biosynthesize silver nanoparticles from Iraqi truffles without heating, and to characterize the biosynthesized AgNPs using the changing color, UV-Visible spectrum, FT-IR, AFM and SEM analyses also to investigate the bioactivity of AgNPs against some pathogenic bacteria *in vitro*.

2. Materials and Methods

2.1. Desert Truffles Samples

Ascocarps (fruiting bodies) of Iraqi desert truffle *Terminia* sp. were obtained from Hit market, Iraq. The ascocarps (Figure 1) were cleaned, sliced, dried in the direct sun at 30-35 °C until stability of weight, and were

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ground to obtain powder. The truffle powder was kept at room temperature in a dry place until use.

Figure 1 Slices of Ascocarps of Iraqi desert truffle *Terminia* sp.

2.2. Bacterial Isolates

Four human pathogenic bacterial isolates were obtained from Al-Ramadi Hospital Lab in Iraq to investigate the bioactivity (antibacterial activity) *in vitro* of the synthesized silver nanoparticles using agar-well diffusion testing. They are *Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella* spp., and *Staphylococcus aureus*.

2.3. Extraction of Desert Truffle Ascocarps

Ten grams of powder of the dried *Terminia* sp. desert truffle with 200 ml distilled water were extracted by stirring on magnetic starrier for sixty minutes to get the crude extract. The crude aqueous extract was filtered using filter paper Whatman No. 1, centrifuged at 4000 cycle/min for fifteen minutes and the aqueous extract was kept at 2 °C for future studies, while the residue was omitted.

2.4. Biosynthesis and Characterization of Silver Nanoparticles Using Terminia sp.

Ten milliliters of *Terminia* sp. desert truffle extract (concentrations 5, 10, 15 mg/ml) and 5 ml of 10^{-3} M $AgNO_3$ were mixed in glass test tubes separately and incubated at 30 °C for twenty-seven hours in a dark place. Changing in color, UV-Visible spectrum, FT-IR, SEM, and AFM analyses were performed to characterize the synthesized silver nanoparticles from desert truffles.

2.5. Antibacterial Activity of the Truffle-AgNPs

The mycosynthesized Ag nanoparticles using *Terminia* sp. desert truffle were tested the antibacterial action by agar-well diffusion testing against the Gram-negative bacteria (*Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella* spp.) and Gram-positive bacteria (*Staphylococcus aureus*). The tested pathogenic bacteria were maintained in nutrient broth for 24 hr and kept undisturbed until use. Muller-Hinton agar plates were prepared for this test. Then 100 µL of 24 hr activated bacterial cultures of individual organisms were spread over Muller-Hinton plate using a cotton swab. Wells of seven millimeters (diameter) were made on each dish by sterile cork borer. Then, 50 µL per well of the biosynthesized Ag nanoparticles solution was poured, and 25 µg/well of Gentamycin was used as a control in this work. The plates were left in a cool place for 30 min to spread the biomaterials then incubated at 37 °C for 24 hr. Zone of inhibition of each bacterium was recorded.

2.6. Statistical Aspect

The data of zones of inhibition of the antibacterial activity test were analyzed in one way analysis of variance by table of ANOVA (SAS program version 9, SAS Institute Inc., USA) Significance of differences were calculated by DMRT (Duncan's Multiple Range Test). Probability value < 5% (p<0.05) is considered to be statistically significant.

3. Results and Discussion

3.1. Characterization of Truffle-AgNPs

The visual observation of color changes from pale yellow to dark brown as shown in Figure 2 after seventy-two hours of incubation in a dark place at 30 °C showed the formation of desert truffle silver nanoparticles (truffle-AgNPs). The synthesis of AgNPs was confirmed by UV-Visible spectra which agree with the results of Owaid *et al*. (2015). Figure 3 showed higher UV-visible peak of 415 nm which confirmed the formation of truffle-AgNPs in this test.

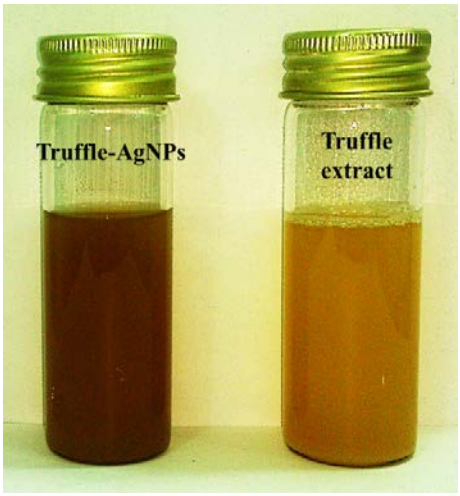

Figure 2. Changing the color of truffle extract with $AgNO_3$ solution after 72 hr

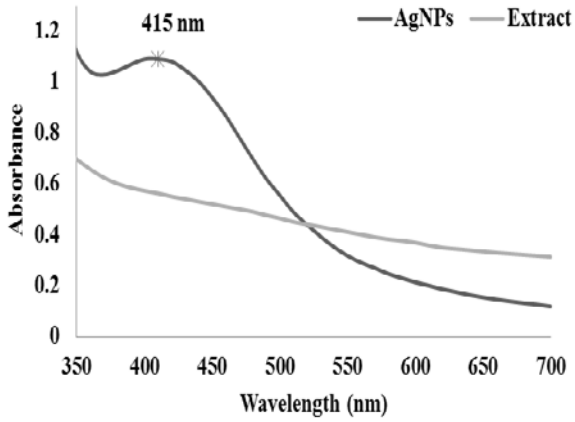

Figure 3, UV-Visible spectrum of the desert truffle-AgNPs solution

The surface topography was investigated using Atomic Force Electron microscopy (AFM) image (Figure 4) which shows the histogram of the percentage of AgNPs as a

function of the grain size. The AFM images illustrate the surface morphology and roughness. Figure 4 shows that the synthesis leads to the formation of spherical, irregular and hummock-like structures in the silver layers. The formation may be connected with an enhanced diffusion of silver particles during their aggregation into more massive structures. Furthermore, SEM image showed an aggregate of dispersed particles of AgNPs as in Figure 5.

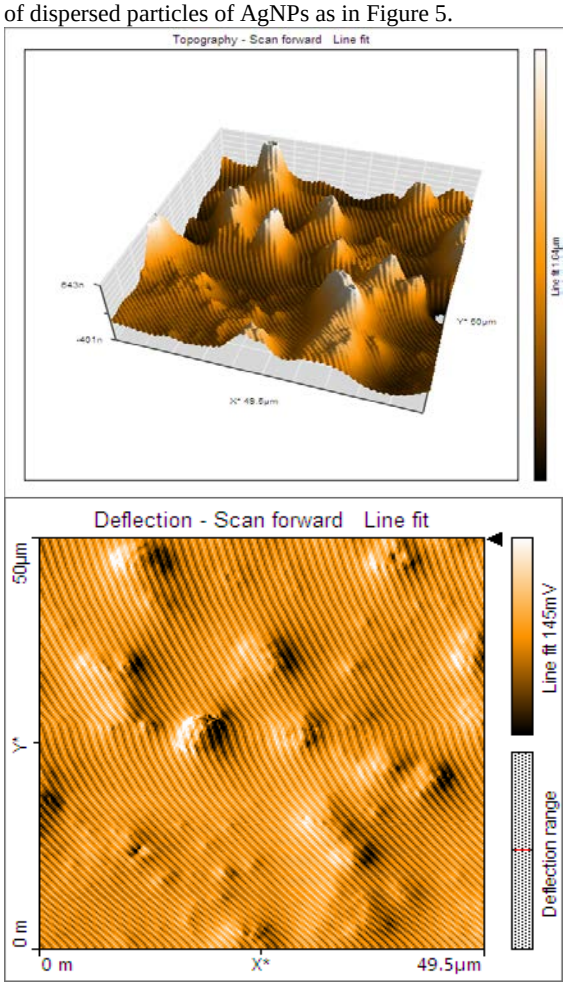

Figure 4. AFM of desert truffle-AgNPs

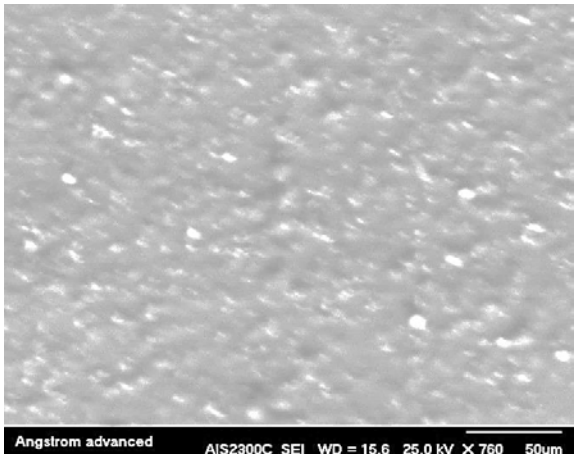

Figure 5. SEM of desert truffle-AgNPs

The FT-IR spectrum of silver nanoparticles was used to identify the potential bio-reductants of the aqueous extract of *Terminia* sp. involved in the reduction of silver nitrate (10^{-3} M). Absorbance peaks from 3461.99 cm^{-1} to 445.53 cm^{-1} represent the role of different functional groups in the bio-reduction of $AgNO_3$ (Figure 6).

The absorbance peaks at 3461.99, 3431.13, 3411.84, 3388.70 and 3290.33 cm^{-1} correspond to a stretch group hydroxyl (O-H). Peaks between 2931.60 and 1969 cm^{-1} correspond to a C=O vibration at the α- and β-unsaturated aldehydes, while the peak 1636 cm^{-1} indicates C=O stretch. Peaks of 1094 cm^{-1} and 611 cm^{-1} are strong indications of heterocyclic compounds such as alkaloids (Meghwal and Goswami, 2012).

In the current study, peaks of 1094 cm^{-1} and 1261 cm^{-1} disappeared after the nanoparticles synthesis indicating the involvement of amine group (C-N) and alcohol group (C-O) respectively in the bio-reduction process. The wide range 2400-2600 cm^{-1} indicates the presence of a carboxylic group (-COOH). The carboxylic group indicates the presence of free amino acids and proteins as well as the presence of fatty acids, but may be small in comparison to the amino acids and proteins. The presence of the peak at 3411 cm^{-1} and the existence of lactam group (-NH-CO-) at the peak of 1631 cm^{-1} are considered clear evidences of the existence of proteins. The lactam group is called amid group which links the different amino acids to make up the protein. The wideband range (2900-3600 cm^{-1}) indicates a hydroxyl group (OH), which means the presence of Tyrosine, Serine, and Threonine, or at least one of them. The peaks of 923 cm^{-1}, 1031 cm^{-1} and 1078 cm^{-1} report the existence of CS CN and CO groups which also indicates the presence of proteins and amino acids. The two packs at 2517 and 2768 cm^{-1} are clear evidence on the existence of -SH group, and this is another evidence of the presence of Cysteine and is likely to be free or existing at the end of the protein.

In addition, the researchers believe there are lipoproteins and lipopolysaccharides but in small amounts. Peak 1373 cm^{-1} indicates the presence of methyl group (CH_3) and is present in the composition of some free amino acids or proteins. The three consecutive peaks at 1147, 1201 and 1244 cm^{-1} are clear and definitive evidences on the presence of the methyl group in the composition of lipids because of the saturated and unsaturated fatty acids starting with its chemical composition by an analog group. The peak at 2931 cm^{-1} indicates the presence of the group (CH) of amino acids, proteins or lipids. Peaks from 636 to 707 cm^{-1} are indicative of the out-of-level curve of the group -N-H. The peaks of 445-559 cm^{-1} indicate that single bonds are connecting an organic compound with individual elements (maybe silver) in the biosynthesized silver nanoparticles, but the type of the element could not be determined here by FT-IR alone (Silverstein *et al*., 2005).

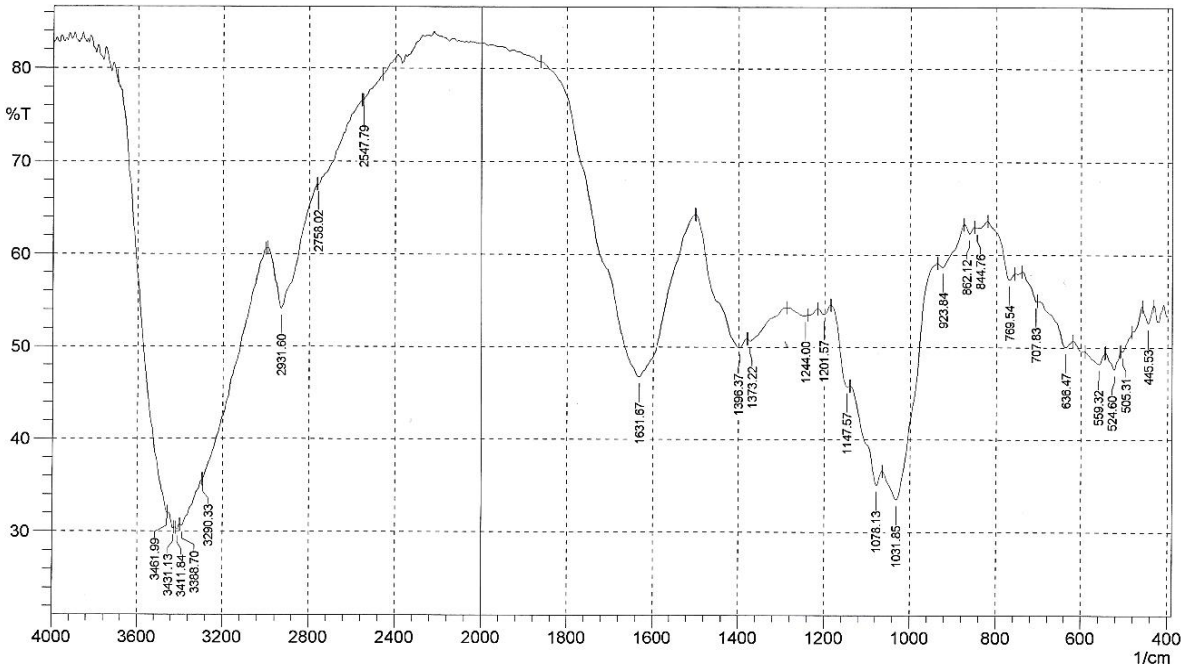

Figure 6. FT-IR spectrum of truffle-AgNPs

3.2. Antibacterial Activity of Truffle-AgNPs

The concentration 2.5 mg/well did not exhibit any inhibitory efficacy as shown in Figure 7. The level 5 mg/well had the best zone of inhibition 10.5 mm against *Pseudomonas aeruginosa* (Gram-negative bacterium) followed by *Staphylococcus aureus* (Gram-positive bacterium) of 9 mm. However, the higher concentration 10 mg/well showed an inhibitory effect against all the studied pathogenic bacteria. The higher inhibition was 14 mm against *Pseudomonas aeruginosa* compared with 31 mm by the control (50 µg/well of gentamycin), followed by *Staphylococcus aureus* (12 mm compared with 25 mm by the control). As shown in Figure 8, the zone of inhibition of *Escherichia coli* and *Klebsiella* spp. recorded 11 and 9.5 mm in the case using 10 mg/well of truffle-AgNPs compared with the control (27 and 24 mm) respectively. The effectiveness of the synthesized AgNPs (as antimicrobial agents) is related to their ratio of high surface area to volume. This property is enabling specific interactions with the bacterial cell membrane (Morones *et al.,* 2005). The truffle-silver nanoparticles play an inhibitory role against the pathogenic bacterium which opens the door towards using this biomaterial (desert truffle) as reducing and stabilizing agents in producing green nano-drugs against human pathogenic bacteria, in particular *P. aeruginosa*.

As the AgNPs come in contact with the bacteria, they adhere to the cell wall and cell membrane. Some of the silver adheres to the sulfur-containing proteins on the membrane (Klasen, 2000). The silver-sulfur interaction at the membrane causes many structural and morphological changes in the cell wall such as the formation of pores and pits. Through these pores, cellular components are released into the extracellular fluid, just due to the osmotic difference. While another portion passes to the inside of bacteria and interacts with DNA and RNA thus AgNPs will inhibit the cell's replication proteins that lead to the death of bacterial cells (Feng *et al*., 2000). This interaction has been linked to the suppression of enzymes, and inhibited the expression of proteins that relate to the ability of cells to produce ATP (Yamanaka *et al.*, 2005).

As the AgNPs interact with the microbes, they adhere to the wall and membrane of cells. A portion of the silver atoms adheres to the sulfur-containing proteins on the cell membrane (Klasen, 2000). The silver-sulfur interaction at the membrane causes numerous basic and morphological changes in the cell wall, for example, the formation and development of pores and pits. Through these pores, some cellular components are discharged into the extracellular fluid, just due to the osmotic difference. While another portion passes to the inside of the bacteria and interacts with DNA and RNA thus AgNPs will inhibit the cell's replication proteins that lead to the death of bacterial cells (Feng *et al.*, 2000). This interaction has been linked to the suppression of enzymes and inhibited the expression of proteins that relate to the ability of cells to produce ATP (Yamanaka *et al*., 2005).

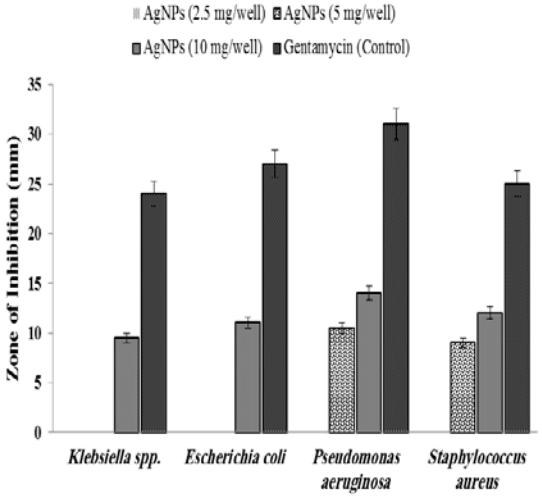

Figure 7. Antimicrobial activities of desert truffle-AgNPs

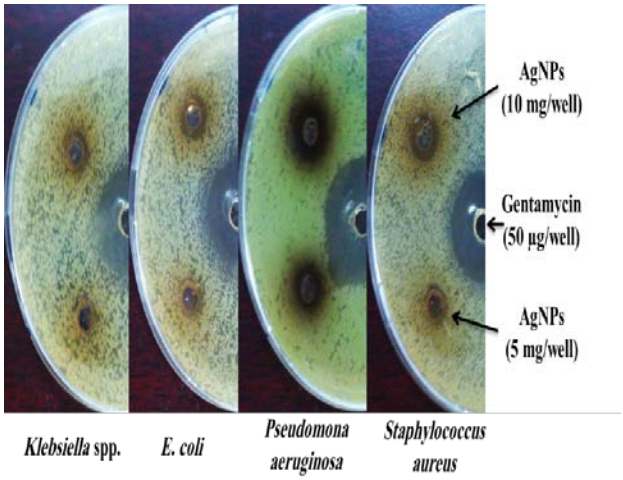

Figure 8. Zone of inhibition of desert truffle-AgNPs by agar-well diffusion testing

4. Conclusion

Desert truffle *Tirmania* sp. is suitable as a greener-reducing agent to mycosynthesize silver nanoparticles at the room temperature in a dark place using the aqueous extract of its ascocarps and $10^{-3}AgNO_3$ solution. The changing in color from pale yellow to dark brown after seventy-two hours is considered a first indicator of the formation of AgNPs. The characterization of those nanoparticles was also achieved by UV-Visible spectrum, FT-IR, AFM and SEM analyses. The UV-visible spectrum showed the formation of AgNPs at peak 415 nm. The AFM and SEM images exhibited silver nanoparticles with spherical or irregular-shaped, agglomerated and highly heterogeneous. The higher inhibitory effect was 14 mm against *Pseudomonas aeruginosa* at the concentration of 5 mg/well. These *Tirmania*-AgNPs have exhibited zones of inhibition of gram-negative and gram-positive bacteria especially the infectious bacteria of the eyes *Pseudomonas aeruginosa*.

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JJBS

Volume 11, Number 4, September 2018
ISSN 1995-6673
Pages 407 - 418

Jordan Journal of Biological Sciences

Vegetation Analysis and Species Distribution in the Lower Tributaries of Wadi Qena in the Eastern Desert of Egypt

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Received January11, 2018; Revised February 21, 2018; Accepted March 7, 2018

Abstract

Vegetation composition and soil attributes in the lower tributaries of Wadi Qena in the Eastern Desert of Egypt are studied from fifty-one stands in three wadis, namely Wadi El-Ghuza, Wadi Naq El-Teir, and Wadi El-Atrash. Altogether, fifty-two species from twenty families and thirty-nine genera, mainly of the Saharo-Arabian focus of distribution, were recorded. Chamaephytes and therophytes constituted 73 % of the main bulk of life form the spectrum of the recorded flora. After the removal of unicates, the presence/absence datasets of thirty-six species and fifty-one stands were classified by TWINSPAN yielding four vegetation groups: *Zygophyllum coccineum-Zilla spinosa-Calligonum polygonoides* (group A) mainly in Wadi Naq El-Teir, *Zygophyllum coccineum-Zilla spinosa* (group B) in Wadi El-Ghuza, *Morettia philaeana* (group C) from the southern part of Wadi El-Atrash, and *Zygophyllum coccineum-Morettia philaeana* (group D) from the northern part of Wadi El-Atrash. These groups were clearly separated along the first two axes of DCA. Soil-vegetation correlations performed by Redundancy Analysis (RDA) indicated that axis 1 was shaped by calcium and organic matter, while axis two was controlled by pH and phosphate contents. Variations of species richness and Shannon diversity index within the separated TWINSPAN groups were highly significant.

Keywords: Vegetation analysis, Arid ecosystems, Soil-environment relationships, Inland wadis, Distribution patterns, Egypt.

1. Introduction

The Eastern Desert of Egypt occupies the area extending from the Nile Valley eastward to the Gulf of Suez and the Red Sea which is about 223,000 km^2, i.e., 21 % of the total area of Egypt. It consists essentially of high back bone of high rugged mountains running parallel to and at a relatively short distance from the coast. These mountains are flanked to the north and west by intensively dissected sedimentary plateau (Said, 1962). Mountains of the eastern desert are of two types: igneous and limestone. The igneous mountains extend southward from about Lat. 28^o N to beyond the Sudano-Egyptian borders at Lat. 22^o N. To the north of the igneous mountains are the extensive and lofty limestone mountains of North Galala, South Galala, and Gebel Ataqa separated by a broad wadi. One of the main features of this desert is that it is intersected by numerous canyon-like depressions (wadis) running to the Red Sea or to the Nile Valley.

The wadis are unique intrazonal landscapes in arid and semi-arid regions of the world (Fossati *et al.*, 1998), as they represent one of the most prominent desert landforms, which exhibit physiographic irregularities that lead to parallel variations in species distribution (Kassas and Girgis, 1964). These wadis are drainage systems for collecting water from extensive catchments areas such as hills, cliffs, slopes, etc. Accordingly, the water supply of a wadi is many times the recorded rainfall with richer vegetation than other types of desert habitat. This advantage is, however, counterbalanced by two destructive agents: torrents and grazing. The water way of the torrents is usually devoid of plant cover, which is restricted to the wadi sides. The influence of torrents on plants is partly mechanical, destroying and uprooting the plants, and partly erosion removing the soil (Kassas and El-Abyad, 1962). In addition, certain species are subjected to serious grazing by animals, while woody plants are liable for cutting as a source of fuel. The development of wadi bed includes the gradual accumulation of transported material. The soil barren bed allows the growth of chaemophytes, while a shallow soil cover, moistened during the rainy season, allows the appearance of the ephemerals. When a deep soil is accumulated allowing the establishment of a wet-soil layer, perennials will find it favourable for their growth. At an advanced stage, deep alluvial deposits allow free water to be stored in the subsoil and a water table is established. At this stage shrubs and/or trees are characteristic of the habitat

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Since Schweinfurth (1901), the botanical exploration of the wadis of the Eastern Desert attracted many botanists and geographers. Ever since, wealth of monumental and documentary works concerned with the flora, vegetation and habitats of the Eastern Desert were published (Abd El-Ghani *et al.*, 2014). The floristics, chorological affinities, plant communities, functional groups, and their environmental relationships received much attention, and yielded several publications in the past few years (Salama *et al.*, 2013, 2014, 2015).

The phytosociological study on the deltaic part and the principal channel of Wadi Qena has been investigated by Salama and Fayed (1990). In the last decades, Wadi Qena was affected by human activities including; cultivation of the deltaic part, the intensive collection of plant species for its values (medicinal, fuel, fibers…etc.), establishment of new settlements and high ways, etc. Undoubtedly, these activities affect the natural flora and vegetation, and changed the distribution of plant species in Wadi Qena. This wadi represents a rather complex heterogeneous ecosystem necessitating arbitrary dismantling on investigation. Due to the variety of chances of water-feeding of its tributaries, it was found appropriate to investigate them in more or less homogeneous groups, apart from the main trunk of the wadi, which collects such waters into a vast delta pouring directly south to the Nile Valley, shortly north east of the city of Qena.

Therefore, the present study was designed to answer the following questions: (1) What is the present status of the floristic composition of the lower tributaries of Wadi Qena (Wadi El-Ghuza, Wadi Naq El-Teir, and Wadi El-Atrash)? (2) What are the plant communities inhabiting different habitats of these wadis? (3) What are the major environmental gradients associated with the species distribution and plant communities in these wadis? and (4) What are the factors affecting the diversity of plant communities inhabiting the study area? These objectives were addressed by applying multivariate analyses methods to the data of species composition and soil environment from fifty-one sample plots.

2. Material and Methods

2.1. Study Area

Wadi Qena (between latitudes 26° 10´ and 28° 00´N) is one of the most notable features of the Eastern Desert of Egypt. The north-south course of its principal channel is unique. It is the largest and greatest dry valley which runs in this desert for a distance of about 220 km. It runs from north to south (i.e., in an opposite direction to the Nile Valley) and debouches at the city of Qena 600 km south of Cairo (Figure 1). Its width varies from 5 to 25 km, and its widest part lies north of Gebel Abu Had. Although this wadi is generally dry all over the year, some seasonal rainfall is experienced in winter time which may occasionally (not regularly) become torrential in autumn and spring times. The torrential rains (in January, 1980 and November, 1996) that swept suddenly over a limited area in the Eastern Desert facing Qena Province (Upper Egypt) resulted in enriching the vegetation of some extremely dry wadis at this location. This leads to the prevalence of annuals and the flourishing of scarce perennial vegetation (El-Sharkawi *et al.*, 1982a, 1982b).

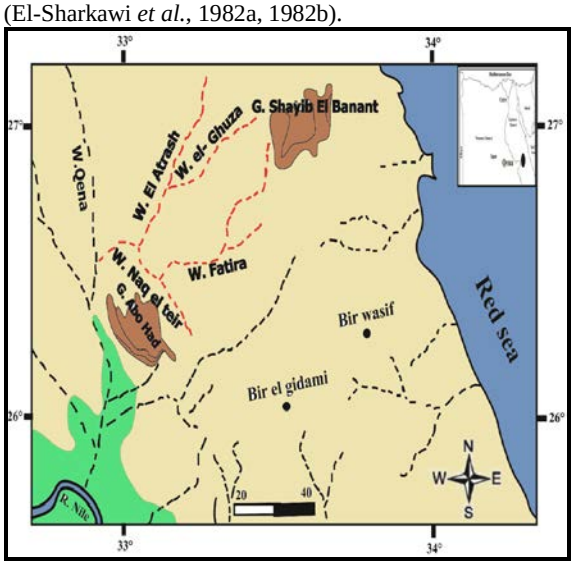

Figure 1. Location map of the 4 studied wadis

Unlike other wadis of the Eastern Desert of Egypt, the main trunk of Wadi Qena has a unique north-south orientation extending for a distance of about 220 km. Its tributaries cover an area between longitudes 32° 10´ and 33° 30´E (Figure 1). The main wadis which debouch their water in Wadi Qena are: Wadi El-Qreiya, Wadi Naq El-Teir, Wadi Fatira, Wadi Hammad and Wadi Zubeir from the east and Wadi Gurdi is the main of the western tributaries. Accordingly, the catchment area of this wadi is immense. Its tributaries, therefore, are not of equal opportunity in receiving waters of sporadic rain. Due to the variety of chances of water feeding of the tributaries of Wadi Qena originating in the mountain range of the Red Sea and pouring their flood waters into its principle channels, difference in floral characteristics and vegetation composition are somewhat expectable.

The scarp around Wadi Qena belongs to the upper Cretaceous-Lower Tertiary rocks. Available meteorological records from the South Valley University station at Qena showed that the average maximum summer temperature was 40.9 °C and the minimum temperature was 25.1 °C. The maximum temperature in the coldest winter month was 23.3 °C and the minimum temperature was 8 °C. Relative humidity ranges from 45.9 % in winter to 17.1 % in summer. Rainfall is negligible occurring only in May with 0.5 mm.

2.2. Vegetation Data Collection

This study was carried out over two successive years, 2014 and 2015. Fifty-one geo-referenced randomly chosen stands (20×20m) were studied in the main wadis where considerable vegetation cover was encountered. These wadis were Wadi El-Ghuza, Wadi Naq El-Teir and lower tributary of Wadi El-Atrash (Figure 1), located between latitudes of 26° 38' and 26° 57'N with mean elevation ranging from 254 m to 498 m above the sea level. The presence and absence of species were recorded from each stand, and a presence percentage (P %) for each species was calculated. The recorded species were classified according to their life forms (Raunkiaer, 1934). The number of species within each life form was expressed as a

percentage of the total number of species in the study area. Taxonomic nomenclature was according to Täckholm (1974), Boulos (1995, 1999, 2000) and El-Hadidi and Fayed (1995). Analysis of phytogeographical ranges of each species follows Zohary (1966, 1972) and Feinbrun-Dothan, 1978).

2.3. Soil Sampling and Analysis

Three soil samples (0-50 cm) were collected from each of the stands, pooled into one composite sample and were left to air drying. Different fractions of sandy soil were separated by the dry sieving method (Ryan *et al.*, 1996). Calcium carbonate ($CaCO_3$) was determined according to the titration method after Jackson (1967). The organic matter contents of the soil samples were determined by loss on ignition methods (Sparks *et al.*, 1996). Water content was determined by weighing the fresh soil sample, drying it in an oven at 105˚C for twenty-four hours, then the dry weight was determined. Sodium and potassium ions were determined by flame photometry according to Williams and Twine (1960), while calcium and magnesium were determined volumetrically by the versene titration method described by Johnson and Ulrich (1959). Chlorides were volumetrically determined as AgCl according to Jackson (1967), sulfates by turbidimetry according to Bardsley and Lancaster (1965), phosphates were determined colourimetrically according to Vogler (1965), and carbonates were estimated by titration using the method described by Jackson (1967). Electric conductivity (EC) of the clear soil filtrate was determined using the conductivity meter according to Jackson (1967). Electric pH-meter was used to determine the soil reaction of the collected samples.

2.4. Multivariate Analyses

Classification and ordination techniques were used to analyze the vegetation. Species recorded in one stands (unicates) were removed from analysis to avoid distortion. So, a presence/absence data matrix of fifty-one stands × 36 species was used and subjected to classification by Two-Way Indicator Species Analysis (TWINSPAN) using the default settings of the computer program CAP (Community Analysis Package) (version 1.2) for Windows (Henderson and Seaby, 1999), and a dendrogram was elaborated. All ordination procedures were performed with CANOCO software (version 4.5) (Ter Braak, 1987, 1990). Detrended Correspondence Analysis (DCA) estimated the compositional gradient in the vegetation data to be ranged from 2.89 to 2.50 S.D units for most subset analysis. Therefore, Redundancy Analysis (RDA) was used as the appropriate direct analysis to measure the soil-vegetation relationships (Ter Braak and Prentice, 1988). Prior to RDA analysis, all data variables were assessed for normality (SPSS version 10.0 for Windows), and appropriate transformations were performed when necessary (Zar, 1984). Six soil variables (sand, clay, electrical conductivity (EC), potassium, sodium and sulphates) were eliminated from the RDA analysis due to the high collinearity (Kutner *et al.*, 2004), and the inflation factor did not exceed 7.5. Therefore, nine soil variables were used in the RDA analysis: gravel, silt, pH, organic matter (OM), water content (WC), calcium, magnesium, phosphates and chlorides. A Monte Carlo permutation test (499 permutations) was used to test for significance of the eigenvalues of the first canonical axis. The TWINSPAN vegetation groups were subjected to ANOVA (One-Way Analysis of variance) based on the soil variables to investigate whether there were significant variations among the groups.

2.5. Species Diversity

Two species diversity measures were employed. Species richness (SR) within each separated TWINSPAN vegetation group was calculated as the average number of species per stand. The Shannon-Wiener diversity index (H') was calculated according to the formula: $H' = -\Sigma_i^S P_i \log_2 P_i$ (Pielou, 1975), where S is the total number of species and P_i is the presence percentage of the i th species.

2.6. Life Forms and Soil Relationship

After classifying these species within their vegetation groups, the number of species within each life form was expressed as a percentage of the total number of species in the vegetation group. DCA estimated the length of gradient in the floristic data, and was 4.12 SD units. Then a data-set of the species life forms (presence or absence) with the soil variables for each stand was analysed by the Canonical Corresponding Analysis (Leps and Šmilauer, 2003) to estimate the correlations between each life form and the soil variables. After eliminating three soil variables (clay, electrical conductivity and potassium) due to their high collinearity, twelve soil variables were used in the CCA analysis: gravel, silt, FS, CS, pH, OM, WC, calcium, magnesium, sodium, phosphates and chlorides. Monte Carlo permutation test (499 permutations) was also used in this analysis. Percentages of four life forms within four TWINSPAN groups were subjected to ANOVA to find out the degree of significance among the groups (Sokal and Rohlfs, 1981).

3. Results

3.1. Floristic Composition

A total of fifty-two species from twenty families of the vascular plants and thirty-nine genera were recorded. Largest families with the highest numbers of species were Zygophyllaceae (nine species), Brassicaceae (six species), Fabaceae and Asteraceae (five species for each), Chenopodiaceae (four species) and Boraginaceae and Resedaceae (three species for each). These families constituted the main bulk of the total flora (thirty-five species or 67.3 %). Analysis of the life form spectrum showed chamaephytes, and therophytes were equally distributed and constituted the main bulk (36.5 % for each) of the recorded flora, while phanerophytes were very scarce (9.6 %). *Fagonia* was the largest genus (four species), whereas the remaining genera were represented by 1-2 species. The majority of the recorded species belong to the Mediterranean and Saharo-Arabian chorotypes.

3.2. Classification of Vegetation

Four vegetation groups were separated after the application of TWINSPAN analysis to the presence/absence data set of thirty-six species recorded in fifty-one stands (Figure 2, Table 1). These groups included

five dominant species that have the highest presence percentages (P %) and were recorded in all groups; viz., *Zygophyllum coccineum, Zilla spinosa, Calligonum polygonoides, Aerva javanica* and *Acacia tortilis* subsp. *raddiana*. Five species showed consistency:

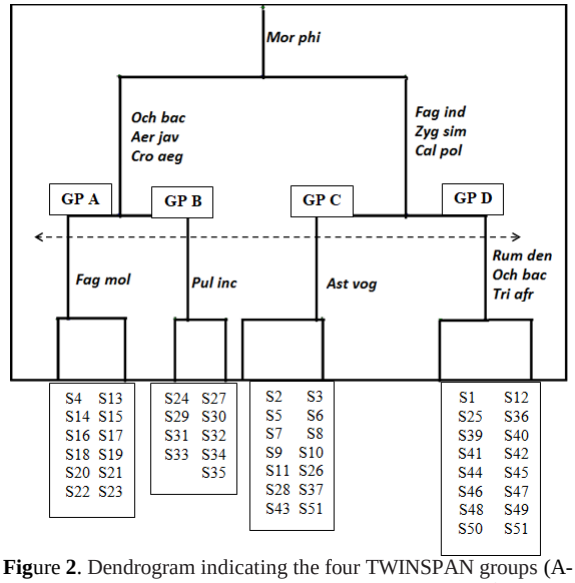

Figure 2. Dendrogram indicating the four TWINSPAN groups (A-D), together with their indicator species resulted from the classification of the 51 stands. For species abbreviations, see Appendix.

group (D) *Rumex dentatus, Tribulus pentandrus, T. longipetalus, Pergularia tomentosa* and *Plantago ciliata*. Notably, groups (C) and (D) share ten species (Table 1) with a lower presence percentages (P = 6.2-37.5 %), whereas *Zygophyllum simplex* and *Forsskaolea tenacissima* attained their highest records (93.7 % for the former and 62.5 % for the latter) in group D.

Similar observation can be noted to another eight species shared groups (B), (C) and (D), but with highest (P %) for *Faginia indica* and *Pulicaria incisa* in group (D). The soils inhabited the fifty-one studied stands were alkaline in nature with pH ranged between 8.57 in group (A) and 8.83 in group (D). Variations between groups showed that sand fractions were the only soil parameter that caused significant difference (p = 0.018).

Group (A) included eleven species from twelve stands collected from Wadi Naq El-Teir (Table 1), and was the least diversified among the other groups. Dominant species were *Zygophyllum coccineum, Zilla spinosa, Calligonum polygonoides*, and co-dominated by *Crotalaria aegyptiaca*. Soil of this group (Table 2) was characterized by the highest contents of sand, sodium and chloride ions, but attained the lowest values of several soil parameters (e.g., organic matter, water content, electrical conductivity, etc.). The fifteen species of group (B) that were sampled from nine stands from Wadi El-Ghuza were dominated by *Zygophyllum coccineum* and *Zilla spinosa*, and co-dominated by *Calligonum polygonoides* and *Aerva javanica*. The soil of this group showed the highest contents of water, electrical conductivity, gravel, calcium and magnesium ions (Table 2), and lower contents of sodium and phosphates. *Morettia philaeana* dominated (P = 100 %) the fourteen stands of group (C) which was sampled from the southern part of Wadi El-Atrash. *Zygophyllum coccineum, Zilla spinosa* and *Calligonum polygonoides* were the co-dominant species with P = 92.8 % for each (Table 1). The stands of this group had the highest values of soil organic matter, and lower contents of other variables. Group (D) was the most diversified among the others (thirty-six species). This group was dominated by *Zygophyllum coccineum* and *Morettia philaeana*, and co-dominated by *Fagonia indica* and *Zygophyllum simplex*. Important but not dominant species included *Forsskaolea tenacissima* and *Stipagrostis plumosa*. Sporadic species included *Seriphidium herba-alba, Crotalaria aegyptiaca, Eremobium aegyptium* and *Diplotaxis acris*. The stands of this group inhabited soil with highest contents of fine sediments (silt and clay), sulphates, pH, sand fractions, and the lowest contents of calcium and potassium ions (Table 2).

Table 1. Floristic composition in the TWINSPAN vegetation groups of studied wadis. Full names of and authorities of species abbreviations are presented in the Appendix.

Species Abbreviations	GP A	GP B	GP C	GP D
Number of stands	12	9	14	16
Number of species	11	15	27	35
Zyg coc	100	100	92.8	100
Zil spi	100	100	92.8	93.7
Cal pol	100	88.9	92.8	12.5
Aer jav	25	88.9	35.7	25
Aca sp	16.7	11.1	28.6	31.2
Fag mol	16.7		14.3	12.5
Mor phi	8.3		100	100
Fag ara	8.3		14.3	6.2
Och bac		44.4	64.3	25
Pul inc		22.2	28.6	75
Fag ind		11.1	35.7	93.7
Cit col		11.1	50	25
Sti plu		11.1	7.1	62.5
Lep pyr		11.1	7.1	18.7
Art jud		11.1	7.1	6.2
Hel dig		11.1	7.1	6.2
Cro aeg	91.7			6.2
Art her	41.7			6.2
Hel bac	8.3			12.5
Chr pli		11.1		25
Res pru		11.1	7.1	
Zyg sim			33.3	93.7
Ere aeg			33.3	6.2
For ten			14.3	62.5
Tri afr			14.3	37.5
Ast vog			7.1	25
Lot pla			7.1	18.7
Oli lin			7.1	12.5
Asp ten			7.1	6.2
Bas mur			7.1	6.2
Dip acr			7.1	6.2
Rum den				31.2
Tri pen				31.2
Per tom				18.7
Tri lon				18.7
Pla cil				12.5

Table. 2 Mean values, standard deviations (SD) and ANOVA values of the soil variables in the TWINSPAN vegetation groups (A-D) of the studied wadis.

Soil variables		TWINSPAN groups A			B			C			D			F-value	*P*
pH		8.57	±	0.39	8.79	±	0.51	8.70	±	0.55	8.83	±	0.44	0.752	0.526
Gravel	(%)	3.53	±	4.24	5.31	±	5.50	5.04	±	5.39	4.97	±	6.03	0.259	0.855
Sand		60.56	±	17.32	38.81	±	19.03	51.02	±	22.97	35.66	±	23.73	3.692*	0.018
Silt		34.06	±	18.45	50.76	±	22.73	36.73	±	20.92	51.77	±	22.44	2.432	0.077
Clay		1.85	±	1.21	5.13	±	3.56	7.21	±	5.42	7.60	±	9.82	2.18	0.103
WC		0.08	±	0.02	0.19	±	0.22	0.11	±	0.06	0.14	±	0.10	2.024	0.124
OM		0.86	±	0.51	1.22	±	0.36	1.57	±	0.85	1.56	±	0.90	2.727	0.055
EC	(mS/cm)	1527.97	±	705.35	2341.07	±	2970	1596.87	±	1515	1723.28	±	3520	0.219	0.883
Ca		0.31	±	0.19	0.49	±	0.69	0.33	±	0.35	0.25	±	0.44	0.579	0.632
Mg		0.04	±	0.02	0.08	±	0.07	0.05	±	0.04	0.07	±	0.10	0.712	0.55
Cl		0.34	±	0.06	0.34	±	0.23	0.30	±	0.14	0.30	±	0.13	0.28	0.84
SO_4		0.33	±	0.26	0.33	±	0.39	0.40	±	0.31	0.41	±	0.40	0.184	0.907
PO_4	(mg/g soil)	0.02	±	0.00	0.02	±	0.01	0.04	±	0.02	0.03	±	0.04	1.949	0.135
Na		0.42	±	0.13	0.30	±	0.28	0.34	±	0.29	0.38	±	0.60	0.175	0.913
K		0.32	±	0.10	0.36	±	0.29	0.34	±	0.23	0.30	±	0.22	0.154	0.926
SR		5.25	±	0.75	5.66	±	1.22	8.28	±	3.5	11.87	±	2.12	23.26**	0.001
H’		2.01	±	0.01	2.11	±	0.33	2.57	±	0.65	3.01	±	0.03	20.90**	0.001

OM=organic matter, WC=water content, EC=Electric conductivity, SR=Species richness and H'=Shannon-Wiener index.**= $p < 0.01$, *= $p < 0.05$.

3.3. Stand and Species Ordination

Figure 3 shows the ordination results of the DCA analysis of the floristic data set. The four TWINSPAN groups were separated along the first (eigenvalue = 0.416) and second (eigenvalue = 0.230) DCA axes. Higher eigenvalues of the first DCA axis indicated that it captured the greater proportion of the variation in the species composition among stands. The four DCA axes explained 14 %, 7.8 %, 5.1 % and 3.8 % of the total variation in the species data, respectively.

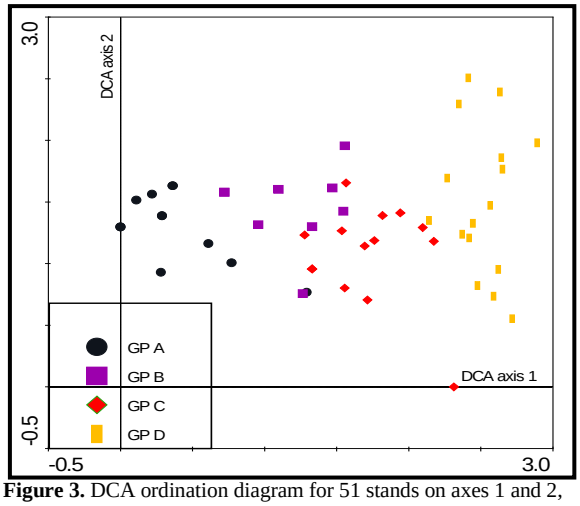

Figure 3. DCA ordination diagram for 51 stands on axes 1 and 2, with the four TWINSPAN groups superimposed.

The lengths of gradients were relatively short: 2.891 for the first axis, and 2.508 for the second. Stands of group (A) were separated toward the negative end of DCA axis 1, while stands of group (D) were separated out along the other end, and those of group (B) and (C) were transitional in their composition between the other groups. DCA axis 1 showed significant positive correlations with organic matter (r = 0.23), water content (r = 0.21) and clay (r = 0.20) and negative correlation with sand (r = -0.28). DCA axis 2 was positively correlated with magnesium (r = 0.26) and pH (r = 0.37) and negatively correlated with PO_4 (r = -0.27). Species ordination by using DCA (Figure 4) revealed the separation of *Calligonum polygonoides*, *Crotalaria aegyptiaca* and *Seriphidium herba-alba*, which characterized group (A), along the negative side of DCA axis 1 from all other species. In the centre of DCA axis 1, *Zygophyllum coccineum* and *Zilla spinosa* occupied an intermediate position along this axis.

3.4. Soil-Vegetation Relationships

Redundancy Analysis (RDA) was used to assess the relationship between the vegetation and soil variables. The TWINSPAN groups (A-D), and the examined soil variables were indicated in the RDA ordination biplot (Figure 5). The species–environment correlations were higher for the first two axes, explaining 60.4 % of the cumulative variance (Table 3).

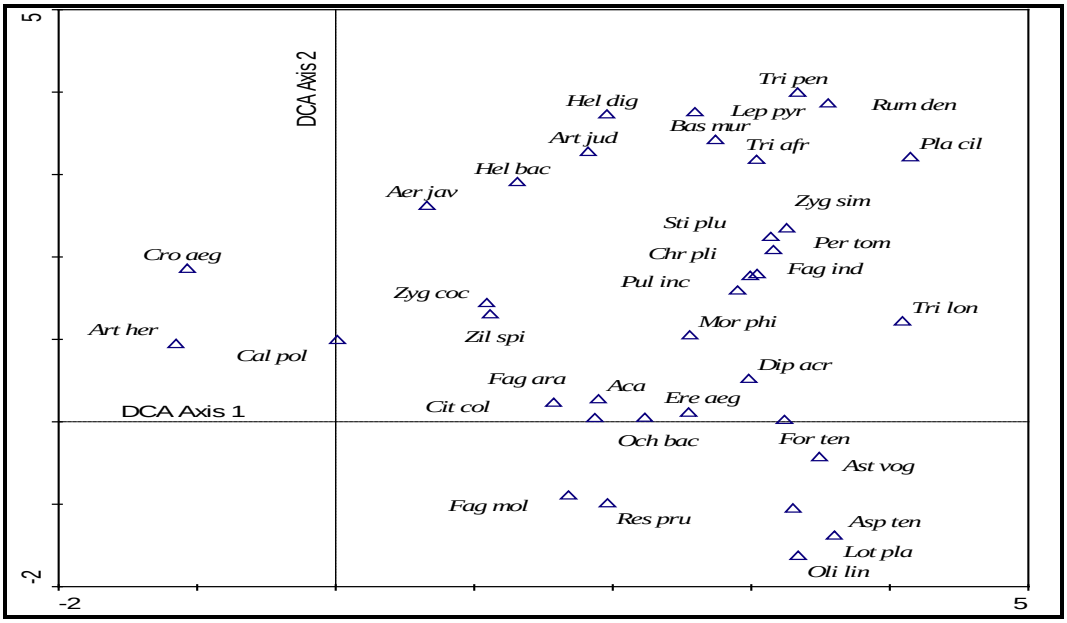

Figure 4. DCA ordination diagram of the 36 species scores on axes 1 and 2. For species abbreviations see Appendix.

The nine soil variables contributed independently to the overall ordination since none of the inflation variables reached higher scores than 15.0 From the interset correlations of the soil factors with the first two axes of RDA (Table 3), it can be inferred that the first axis was negatively correlated with organic matter (r = -0.316) and positively correlated with calcium (r = 0.21). The second axis was defined by pH (r = -0.25) and phosphate contents (r = 0.382). A test for significance with unrestricted Monte Carlo permutation test (499 permutations) indicated that the F-ratio for the eigenvalue of axis 1 and the trace statistic to be significant (p = 0.002). The ordination diagram produced by RDA showed similar pattern to the floristic DCA ordination, with most of the stands remaining in their respective TWINSPAN vegetation groups. It can be noted that stands of groups (A) and (B) occupied the right side of the ordination plane, and were correlated by calcium and chloride ions. On the other hand, the remaining vegetation groups (C and D) occupied the left side of the plane and were correlated by several soil factors such as organic matter, phosphates, sulphates and magnesium ions, pH and gravel.

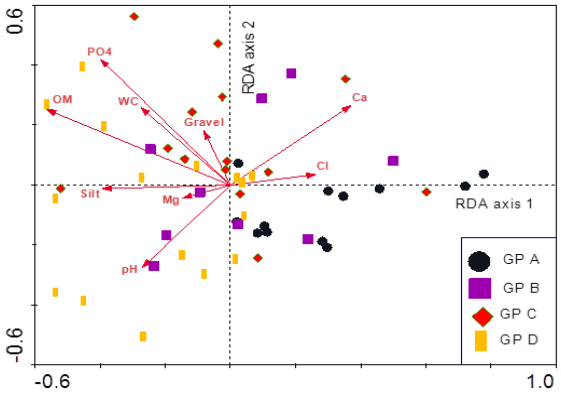

Figure 5. Ordination biplot yielded by Redundancy Analysis (RDA) of the 51 stands with their TWINSPAN groups and soil variables.

Table 3. Interset correlation of the soil variables together with eigenvalues and species–environment correlation along the first 2 RDA axes.

	RDA Axes	
Soil variables	1	2
Eigenvalues	0.09	0.049
Species-environment correlations	0.63	0.74
% Cumulative variance of species data	39.9	60.4
Organic Matter (OM)	-0.316	0.23
Water Content (WC)	-0.153	0.23
Ca	0.21	0.242
Mg	-0.08	-0.040
Gravel	-0.045	0.165
Silt	-0.220	-0.011
Cl	0.146	0.032
PO_4	-0.223	0.382
pH	-0.151	-0.25

3.5. Species Diversity

Both estimated species diversity indices (species richness and Shannon-Wiener index) showed significant variations among the separated TWINSPAN groups (Table 2). However, Pearson correlation coefficients (r) between the examined soil variables and diversity indices exhibited insignificant correlations (results not shown). In addition, group (A) had the lowest species richness (5.25±0.75 species stand^{-1}) and Shannon-Wiener index (2.01±0.01), while group (D) had the highest diversity records.

3.6. Life Forms and Soil Relationships

This relationship was clear between most of the annuals (therophytes) and the soil water content, organic matter, silt, pH, Mg and PO_4. Meanwhile, the soil-surface buds-bearing perennials (hemicryptophytes) were mainly dependent on silt, fine sand, and soil reactions (pH). Also, there was a correlation between the low-growing perennial shrubs (chamaephytes) and the salinity factors (Na and Cl), coarse sand, and fine sand. The woody trees and shrubs (phanerophytes) were concentrated in the upper half of CCA biplot (Figure 6) with organic matter and most estimated soil anions and cations.

The successive decrease of eigenvalues of the two CCA axes (0.206 and 0.158 for axes 1 and 2, respectively), illustrated in Table (4), suggested a well-structured data set. The species environment correlations were high for the first two axes, explaining 81.3 % of the cumulative variance.

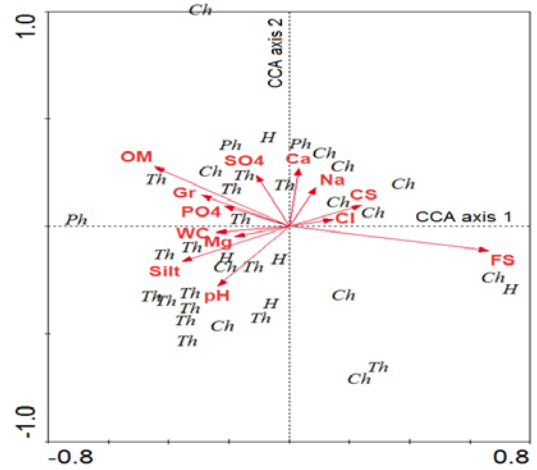

Figure 6. CCA biplot indicating the relationship between life form categories of the studied species and soil variables.

The interset correlations resulted from Canonical Correspondence Analysis (CCA) of the examined soil variables were displayed in Table (4). CCA axis one can be interpreted as fine sand-organic matter gradient; also axis two can be interpreted as organic matter-pH gradient, while the third axis may name PO_4-pH gradient. A test for significance with an unrestricted Monte Carlo permutation test (499 permutation) for the eigenvalue of axis one was found to be significant ($p = 0.03$).

Significant differences (ANOVA) between the percentages of each life form within the separated vegetation groups were demonstrated in Table 2. The variations of hemicryptophytes and therophytes among the four vegetation groups showed high significant differences among groups at $p < 0.01$, while phanerophytes showed significant difference among these groups at $p < 0.05$. However, there was no difference between their chamaephytes. These calculations indicated a significant rise of hemicryptophytes in group D (42.5 %), while the minimum percentage was in group B (8.89 %; Table 2). For phanerophytes; the highest percentage was in group C (30.95 %), and the minimum was in group A (5.56 %). On the other hand, the most represented life form (therophytes) recorded the highest percentage (25.78 %) at group D, but it was nearly not represented in group A (0.52 %).

4. Discussion

As in most of the hyperarid desert environments, the vegetation in the study area is restricted to wadis, runnels and depressions with deep fine sediments that receive an adequate water supply (Abd El-Ghani *et al.*, 2017). Minimal precipitation and frequent droughts characterized the vegetation in the arid regions; therefore the availability of water is one of the primary factors controlling the distribution of species (Yair and Danin, 1980; Salama *et al.*, 2016).

Table 4. Interset correlation of the life forms and soil variables, together with eigenvalues and species–environment correlations along the first 2 CCA axes.

	CCA Axes	
Soil variables	1	2
Eigenvalues	0.206	0.158
Species-environment correlations	0.748	0.813
% Cumulative variance of species data	17.8	31.4
OM	-0.33	0.226
WC	-0.18	-0.02
Ca	0.024	0.22
Mg	-0.14	-0.04
Gravel	-0.22	0.118
CS	0.18	0.08
FS	0.496	-0.09
Silt	-0.27	-0.13
Na	0.066	0.148
Cl	0.111	0.027
PO_4	-0.16	0.077
SO_4	-0.08	0.195
pH	-0.18	-0.23

Parker (1991) suggested that the distribution of the dominant species and variations in distributional patterns over a small geographic area in the desert ecosystem may be related to edaphic factors and local topography. The plant life in the study area is restricted to microenvironments (as in wadis, runnels and depressions), where runoff water collects and provides sufficient moisture for plant growth which is mainly formed of xerophytic shrubs and sub-shrubs to withstand the harsh environmental conditions. The vegetation is mosaic characterized by sparseness of plant cover, a limited number of plant species (mean species richness 8.2±3.5 on the average in studied stands), and paucity of trees (less than 5 % of the recorded species). Mono-dominant stands are not as common as those dominated by more than one species. Walter *et al.* (1975), considered the study area within the subtropical dry zone which has very hot summers and mild winters with a short rainy season. Therefore, the dominant perennial species provide the permanent character of the plant cover. Shortly after rainfall, the appearance of annuals provides a characteristic physiognomy to the vegetation (Alatar *et al.*, 2012).

Chorological analysis of the floristic data revealed that the Saharo-Arabian chorotype forms the major component of the floristic structure where it was represented by more than 50 % in the studied wadis. This can be attributed to the ability of the Saharo-Arabian species to withstand the harsh environmental desert conditions. Similar conclusions were indicated in the Egyptian desert (Salama *et al.*, 2012), in the Saudi Arabia highlands (Abdel Khalik *et al.*, 2013), and in the Libyan Sahara (Hegazy *et al.*, 2011). Preponderance of chamaephytes and therophytes over other life forms may be attributed to the hot dry climate and the continuous anthropogenic effects (Asri, 2003).These results are congruent with the vegetation spectra in other parts of the Middle East (Danin and Orchan, 1990).

The dominance of *Acacia tortilis* subsp. *raddiana* in wadi channels has been reported in many parts of the arid Middle East (Robinson, 2004; Woldewahid *et al.*, 2007) and is supported in this study. It is an important fodder tree

for the livestock and as a source of fuel wood for the Bedouin people. A recent evaluation of the conservation status of this species has pointed to the massive mortality of mature trees, and a corresponding lack of recruitment by young trees (Rohner and Ward, 1999). Therefore, it is believed to be endangered in many parts of the Middle East (Wiegand *et al.*, 1999).

In the different vegetation patterns, plant life forms (Boulos, 1999, 2000, 2002, 2005) showed a characteristic distribution. True annuals (e.g., *Astragalus vogelii*, *Asphodelus tenuifolius*, *Cotula cinerea*) occurred directly after rainfall. Most other species proceed through therophytic, short-lived perennial (*Morettia philaeana*, *Pulicaria incisa*, *Zygophyllum simplex*) or even long-lived perennial life cycles (*Crotalaria aegyptiaca*, *Fagonia* spp., *Pulicaria incisa*, *Zilla spinosa*) depending on the soil moisture content (Springuel *et al.*, 2006). These species with life cycles determined not by an internal but by an external factor are called poikilorhythmic species (Bornkamm, 2001). They are the characteristic life form of accidental vegetation, and were described in parts of the Western Desert of Egypt (Abd El-Ghani, 2000). The xeropsammophytes such as *Fagonia arabica*, *Cornulaca monacantha*, *Zilla spinosa*, *Calligonum polygonoides*, *Pulicaria incisa*, *Citrullus colocynthis*, and *Heliotropium digynum* were found in dry non-saline sandy sites with higher fertility soils, where infiltration is higher and water accumulates in deeper layers. These species are distributed widely in Egypt (Zahran and Willis, 2009) and the neighbouring countries (Wojterski, 1985).

In most of the arid regions, correlation of soils and vegetation were investigated in various habitats through the application of multivariate analyses techniques. In the desert ecosystem García-Novo *et al.* (2004), Enright *et al.* (2005), Salama *et al.* (2016) and van Etten and Fox (2017) worked in this direction. These investigations include large areas, and therefore they reported striking gradients referring to soil conditions and vegetation. In this study, two approaches of multivariate analyses were used. The heterogeneity of microclimatic conditions and the local topography affected the variation of the distributional behaviour of the plant associations in the study area. In terms of vegetation and floristic composition, four vegetation groups (plant communities) were identified and represented the plant communities that characterized the studied wadis: *Zygophyllum coccineum-Zilla spinosa-Calligonum polygonoides* (group A) mainly in Wadi Fatira, *Zygophyllum coccineum-Zilla spinosa* (group B) in Wadi El-Ghuza, *Morettia philaeana* (group C) from the southern part of Wadi El-Atrash, and *Zygophyllum coccineum-Morettia philaeana* (group D) from the northern part of Wadi El-Atrash. Most of the identified vegetation groups have very much in common with that recorded in some wadis vegetation of the Egyptian desert and the neighbouring countries (Tielbörger, 1997; Sheded *et al.*, 2014). Analysis of the vegetation-soil relationships using Redundancy Analysis (RDA) indicated that the distribution of vegetation in the study area was mainly controlled by pH, organic matter, calcium, and phosphate contents. The role of organic carbon as a key element in soil fertility is well known. Sharaf El-Din and Shaltout (1985) pointed out the importance of soil organic matter in delimiting vegetation groups in the bed of Wadi Araba in the northern part of the Eastern Desert. The role of the percentages of surface sediments of different size classes in the spatial distribution of soil moisture was reported in similar investigations (Dasti and Agnew, 1994). The precipitation of silt and clay that are carried by rains and torrents to the studied wadis increased their percentages in the soil texture. The decomposition of plant residues increased the organic matter content in the soil, and the dissolved potassium and calcium that came with rainwaters decreased the sodium toxicity (Taiz and Zeiger, 2002), and lead to enrich the vegetation diversity (Traut, 2005).

Differences in responses of the life forms to their soil variables indicated that the soil water content, organic matter, silt, pH, Mg and PO_4 act as the main environmental requirements to therophytes of this study area. Meanwhile, most of perennials (hemicryptophytes, chamaephytes and phanerophytes) were mainly preferred organic matter, soluble ions especially salinity factors (Na and Cl), coarse sand and fine sand. From this investigation, it was clear that annuals dominated the silty plains of the study area while the others (perennials) prefer sandy soils.

These calculations indicated a significant rise of hemicryptophytes and therophytes in the northern silty-fertile half of Wadi El-Atrash (group D), while their lower representation was in the poor area of Wadi El-Guzah (group B). Phanerophytes went in a similar manner of the previously mentioned life forms, but they increased significantly in the southern half of Wadi El-Atrash (group C) which is rich in OM, SO_4, PO_4 and K. This increase in such life forms in the main trunk of Wadi El-Atrash may be because this Wadi is considered as a main runoff in the rainy seasons which debouch their water in Wadi Qena. On the other hand; the saline soil of group A (Wadi Naq El-Teir) was nearly deprived of the upstory (trees and shrubs) and the annuals. All these results were previously observed by Salma *et al.* (2014) in three transects in the southern quadrate of Eastern Desert between Aswan and Marsa Alam provinces.

5. Conclusions

The local topography and heterogeneity of the microclimatic conditions affected the distributional patterns of the plant associations in the study area. This study has revealed a much richer variety of floristic resources in Wadi El-Ghuza than the others. It has shown that the distribution of its vegetation is more strongly related to physical environmental factors associated with substrate conditions that affect the water supply (silt and organic matter), than to soil salinity factors. Nevertheless, the latter designed the poor vegetation of Wadi Naq El-Teir. This study area had mainly a simple xerophytic floristic composition, mostly of Sahao-Arabian chorotype focus of distribution, thus, they are relatively not affected by human disturbances. Annuals exhibited clear relationship between most of the soil water content, organic matter, silt, pH, Mg and PO_4. It was clear that annuals dominated the silty plains of the study area, while others (perennials) prefer the sandy soils.

Acknowledgements

The authors would like to express their appreciation to a group of anonymous reviewers for their keen revision, valuable suggestions and comments which improved the first version of this paper.

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Appendix

Species	Spp Abb
Acacia tortilis subsp. *raddiana*	Aca
Aerva javanica (Burm. f.) Juss. ex Schult.	Aer jav
Artemisia judaica L.	Art jud
Asphodelus tenuifolius Cav.	Asp ten
Astragalus vogelii (Webb) Bornm.	Ast vog
Bassia indica (Wight) A. J. Scott	Bas ind
Bassia muricata (L.) Asch.	Bas mur
Calligonum polygonoides L.	Cal pol
Chrozophora oblongifolia (Delile) Spreng.	Chr obl
Chrozophora plicata (Vahl) Spreng.	Chr pli
Citrullus colocynthis (L.) Schrad.	Cit col
Cleome arabica L.	Cle ara
Cornulaca monacantha Delile	Cor mon
Cotula cinerea Delile	Cot cin
Crotalaria aegyptiaca Benth.	Cro aeg
Diplotaxis acris (Forssk.) Boiss.	Dip acr
Diplotaxis harra (Forssk.) Boiss.	Dip har
Eremobium aegyptiacum (Spreng.) Asch. & Schweinf. ex Boiss.	Ere aeg
Fagonia arabica L.	Fag ara
Fagonia bruguieri DC.	Fag bru
Fagonia indica Burm.f.	Fag ind
Fagonia mollis Delile	Fag mol
Fagonia thebaica Boiss.	Fag the
Filago desertorum Pomel	Fil des
Forsskaolea tenacissima L.	For ten
Heliotropium bacciferum Forssk.	Hel bac
Heliotropium digynum (Forssk.) C. Chr.	Hel dig
Leptadenia pyrotechnica (Forssk.) Decne.	Lep pyr
Lotononis platycarpa (Viv) Pic.Serm.	Lot pla
Mesembryanthemum nodiflorum L.	Mes nod
Morettia philaeana (Delile) DC.	Mor phi
Ochradenus baccatus Delile	Och bac

Species	Spp Abb
Oligomeris linifolia (Hornem.) J. F. Macbr.	Oli lin
Pergularia tomentosa L.	Per tom
Plantago ciliata Desf.	Pla cil
Polycarpaea repens (Forssk.) Asch. & Schweinf.	Pol rep
Pulicaria incisa (Lam.) DC.	Pul inc
Reseda pruinosa Delile	Res pru
Retama raetam (Forssk.) Webb & Berthel.	Ret rae
Rumex dentatus L.	Rum den
Salsola villosa Schult.	Sal vil

Species	Spp Abb
Seriphidium herba-alba (Asso) Soják	Art her
Sisymbrium irio L.	Sis iri
Stipagrostis plumosa (L.) Munro ex T. Anderson	Sti plu
Tamarix aphylla (L.) H. Karst.	Tam aph
Tamarix nilotica (Ehrenb.) Bunge	Tam nil
Trichodesma africanum (L.) R. Br.	Tri afr
Tribulus longipetalus Viv.	Tri lon
Tribulus pentandrus Forssk.	Tri pen
Zilla spinosa (L.) Prantl	Zil spi
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JJBS

Jordan Journal of Biological Sciences

Volume 11, Number 4, September 2018
ISSN 1995-6673
Pages 419 - 426

The Prospects of the Cultivation of *Arthrospira platensis* under Outdoor Conditions in Malaysia

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Received January 25, 2018; Revised March 4, 2018; Accepted March 10, 2018

Abstract

There is no virtual report on the commercial cultivation of *Arthrospira* in Malaysia beyond the laboratory scale probably because of the high costs of production and the lower yield which are highly interconnected with the algal cultivation techniques. One way to alleviate the production cost is through outdoor mass cultivation under natural conditions using all available resources. Therefore, the present study was conducted to investigate the prospects of the production of *Arthrospira platensis* under Malaysian tropical climate using enhanced cultivation techniques to reach a maximum yield. In this study, the growth and yield of *A. platensis* were investigated under three different cultivation conditions: laboratory (control), outdoor shaded (greenhouse, T1), and outdoor non-shaded (field, T2). The algal growth was measured through optical density, biomass dry weight, and chlorophyll *a* content. The algal yield was determined by calculating its productivity and specific growth rate. The *A. platensis* cultivation under outdoor non-shaded conditions achieved significantly higher growth ($p < 0.05$) with 1.62 ± 0.038 ABS of maximum optical density, 0.88 ± 0.020 g L^{-1} of maximum biomass dry weight, 8.77 ± 0.219 mg L^{-1} of maximum chlorophyll *a* content, 0.091 ± 0.0022 g L^{-1} d^{-1} of productivity and 0.220 ± 0.0017 µ d^{-1} of specific growth rate over a cultivation period of eight days. The present finding showed that the Malaysian climate is suitable for a satisfactory *A. platensis* productivity with proper cultivation techniques such as the pre-adaptation of the algal culture, inoculation in the late evening, continuous agitation and compensation of the evaporated culture medium.

Keywords: *Arthrospira platensis*; Biomass production; Outdoor cultivation; Cultivation techniques; Tropical climate

1. Introduction

Global warming and food insecurity associated with the inevitable increasing world population have been major global issues threatening humanity as a whole, specific societies, the economy, and nature over the past several decades. Human activities such as logging, deforestation, agricultural practices, waste disposal, and extensive usage of fossil fuels such as coal, oil and natural gas increased the emissions of CO_2, which is a major component of the greenhouse gasses responsible for global warming and climate change (Omer, 2008). Anthropogenic activities simultaneously with the escalating climate change resulted in water scarcity, depletion of cultivable land, and soil infertility, the three major bottlenecks hindering productive agricultural practices. Subsequently, failure in global food production to meet the rapidly-growing world population and the increasing demand for food ultimately resulted in global food crisis, starvation, malnutrition, morbidity and mortality. With the growing concerns about the impacts of global warming and food crisis, societies started to look for sustainable, economically-feasible, and environment-friendly technologies to mitigate the effects of global warming and sustain food production around the world. From this perspective, the photoautotrophic cultivation of microalgae gained plausible attention mainly because of its simultaneous CO_2 sequestration and the production of profitable bio-active compounds in a single process (Ravindran *et al*., 2016).

Considering the advantages of microalgal cultivation, research and developments began with commercial-scale production of *Chlorella*, *Scenedesmus*, *Arthrospira* and *Dunaliella* (Pulz and Gross, 2004; Borowitzka, 1999). Among them, *Arthrospira* (previously known as *Spirulina*) has attracted public and private interests due to its peculiar properties such as its large filamentous size (0.5 mm length); good for an easy harvesting, its effortlessly-digestible cell membrane and because it poses less risks regarding the external contamination due to its ability to

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grow in saline environments and alkaline conditions (up to pH 11) unlike other species (Pelizer *et al*., 2003). *Arthrospira* is a photosynthetic, blue-green, spiral-shaped and multicellular cyanobacterium, which has been consumed as food for thousands of years (Jensen *et al*., 2001). This microorganism is recognized as a potential dietary supplement due to its rich nutritional value such as the abundant protein content (50 – 70 % dry weight), low nucleic acid content, vitamins, pigments, minerals, polyunsaturated fatty acids (PUFAs) among others (Habib *et al*., 2008). Nowadays, the industrial production of *Arthrospira* has stepped into its fourth decade. In fact, this biological origin food, commonly known as Spirulina, is magnificently sustained in the market because of its well-established antioxidants, hypocholesterolemic, anti-inflammatory, antiviral, anticancer, as well as its hepatoprotection and immune-enhancing properties which have been confirmed through research findings (Makhlouf and Makhlouf, 2012; Wu and Ho, 2008).

Currently, the global commercial production of *Arthrospira* is estimated to be at about 8,000 metric tons per year (Vonshak *et al*., 2014), which is mainly contributed by China, followed by other producers in North America and the Asia-Pacific region (Belay, 2013). Concerning this low annual yield compared with the escalating global population, numerous studies are being conducted since the past two decades in order to maximize the worldwide *Arthrospira* production mainly through focusing on abiotic factors such as light intensity and wavelength, temperature, medium composition and concentration, pH and salinity in different cultivation systems including open ponds and photobioreactors under both indoor (laboratory) and outdoor conditions. Subsequently, the individual or the combined effects of these environmental factors on the productivity and biomass composition of *Arthrospira* under controlled laboratory conditions have been intensively investigated and well documented. These previous findings postulated that relatively high temperatures and a profuse supply of light are prerequisites for an optimal growth of this blue-green microalga (Belay, 2008).

Being a tropical country, the Malaysian humid climate with its favourable temperatures ranging from 25 °C to 35 °C as well as the high intensity and duration of sunlight could be very advantageous in the cultivation of *Arthrospira* throughout the year given that there is neither winter nor cold seasons. There were few pilot scale studies conducted in both indoor and outdoor conditions of the local climate (Fagiri *et al*., 2013; Phang *et al*., 2000) showing the prospective of the *Arthrospira* cultivation in Malaysia. However, the commercial production of this microalga is not extensively applied in Malaysia up to now due to the lack of know-how and awareness of its potential profitability, the high cost of production and the low biomass yield. Hence, cultivation techniques should be enhanced in order to optimize *Arthrospira* productivity through economical, feasible and applicable approaches. One way to achieve this goal is by growing this blue-green alga under outdoor conditions using natural solar radiation at ambient temperatures (Vonshak, 1997). Outdoor microalgal production is a complex system synergistically affected by a large number of variables including the quantity of solar radiation, photoperiod, fluctuating temperatures, humidity changes, salinity and pH (Borowitzka, 1999). Those who efficiently simulated the natural ecosystems for the *Arthrospira* production by surmounting the problems raised proved successful and sustained in this industry.

Many suggestions, improvements and constraints associated with the outdoor mass production of *Arthrospira* have been discussed before but, these were mostly related to the seasonal regions (Vonshak and Richmond, 1988). Based on the previous investigations, diurnal fluctuations that impose photo-inhibition, light limitation (Lu and Vonshak, 1999), and hot temperatures above 40 °C (Chanawongse *et al*., 1994) during a significant part of the day are considered as major setbacks for outdoor cultivation as they restrain the culture from achieving its maximum productivity by decreasing the photosynthesis activity, which sometimes causes cell death (Chaiklahan *et al*., 2007). In some former studies, outdoor *Arthrospira* cultivation showed a marked decrease in cell density while better growth was observed under laboratory conditions with continuous light (Vonshak and Richmond, 1985) and outdoor shaded conditions (Vonshak and Guy, 1992). There is lack of information on the cultivation of *Arthrospira* in non-shaded outdoor conditions in the Malaysian tropical climate with 12:12 photoperiod where the intensity of sunlight used to be above 1,000 µmol m^{-2} s^{-1}, and the ambient temperature exceeding 40 °C during the midday. Henceforth, the present study was conducted under three different cultivation conditions in laboratory, outdoor shaded and outdoor non-shaded sites to investigate the prospects of the *Arthrospira* cultivation under Malaysian tropical climate using proper techniques to reach the maximum algal yield.

2. Materials and Methods

2.1. Arthrospira platensis Culture

An axenic culture of *A. platensis* was obtained from The Culture Collection of Algae at The University of Texas, Austin (UTEX). This microalgal species was cultured and pre-adapted in indoor and outdoor conditions using standard Kosaric medium through batch cultivation for two months to facilitate the *A. platensis* adaptation to the new environmental conditions.

2.2. Experimental Cultivation Conditions for Optimum A. platensis Growth

The indoor cultivation of *A. platensis* was experimented under controlled conditions in a pure culture room of Plant Physiology Laboratory, Biology Department at the Faculty of Science, Universiti Putra Malaysia (UPM) at 27.5 ± 1 °C with a continuous illumination (24 h photoperiod) of 27 ± 2 µmol m^{-2} s^{-1} using Philips TLD fluorescent light. Concurrently, the outdoor growth study was conducted in shaded and non-shaded cultivation sites under fluctuating environmental conditions with 12:12 photoperiod. Accordingly, the growth of *A. platensis* under shaded outdoor conditions was investigated in a greenhouse located in the Biology Department. Meanwhile, non-shaded outdoor cultivation of this cyanobacterium was experimented in Field 2, Faculty of Agriculture, UPM.

2.3. Preparation of Experimental Growth Medium

Standard Kosaric medium (SKM) was prepared in half concentration as described by Sukumaran *et al*. (2018) as follows (g L^{-1}): 4.500 $NaHCO_3$, 0.250 NaCl, 0.010 $CaCl_2$, 0.050 $MgSO_4 \cdot 7H_2O$, 0.625 $NaNO_3$, 0.125 K_2HPO_4, 0.250 K_2SO_4, 0.025 $FeSO_4 \cdot 7H_2O$ and 0.5 mL L^{-1} of trace metals solution composed of the following elements (g L^{-1}): 2.86 H_3BO_3, 1.81 $MnCl_2 \cdot 4H_2O$, 0.22 $ZnSO_4 \cdot 7H_2O$, 0.08 $CuSO_4 \cdot 5H_2O$, 0.01 MoO_3, and 0.01 $COCl_2 \cdot 6H_2O$.

2.4. Experimental Design

A. platensis cultured under laboratory conditions was considered as control to study the growth and productivity of *A. platensis* in shaded outdoor (greenhouse; T1) and non-shaded outdoor (field; T2) conditions. Control, T1 and T2 were cultured in five replicates in 5 L sterilized polyethylene bottles containing 4 L working volume. Consequently, 10% (v/v), which is 400 mL of the pre-adapted algal culture was transferred into 3.6 L cultivation medium of the respective control and treatments. Two holes were designed on the cap of the bottle to hold tubing for aeration and gas exchange respectively. The algal culture was aerated using an aquarium air pump (80W, 220V - 240V, Hailea®) through standard 3/16-inch diameter airline tubing with an air stone suspended in the middle of the bottle to provide continuous mixing and agitation. The cultivation period was fixed at eight days based on preliminary observation where the algal culture under non-shaded outdoor conditions started to experience a stationary phase on day nine and onwards. The cultivation process was conducted during mostly sunny and clear skies with minimum rainfall weather conditions.

2.5. Measurement of Environmental Factors during A. platensis Cultivation

Throughout the *A. platensis* cultivation period, the environmental parameters including light intensity (µmol m^{-2} s^{-1}) and air temperature (°C) were recorded daily from seven am to seven pm with a two-hour interval period using a light meter (Li-250, LI-COR® Biosciences) and thermometer (Traceable® Big-Digit Four-Alert Alarm, Fisher Scientific) respectively.

2.6. Measurement of A. platensis Growth

The growth of *A. platensis* cultured in control, T1 and T2 was measured using three growth parameters, namely: optical density, biomass dry weight, and chlorophyll *a* content to precisely determine the growth pattern of this microalga. The algal sample was homogenized each time before being analysed to avoid the sedimentation of cells, which could adversely affect the precision of measurements. The optical density of *A. platensis* cultures was measured daily using spectrophotometer (Hitachi U-1900) at 620 nm (Sukumaran *et al*., 2014).

The biomass dry weight of *A. platensis* was determined gravimetrically every alternate day following Lee and Shen (2004). After being filtered with pre-washed, dried and pre-weighed glass microfiber filter papers (GF/C, 47 mm Ø, 1.2 µm pore sizes, Whatman®) under vacuum, the cells were rinsed with distilled water in order to eliminate leftover mineral salts and other possible extracellular material. After removing the excess moisture through a two-stage vacuum pump, the filtered cells were dried in an oven (Memmert) at 60 – 70 °C for twenty-four hours and were then weighed to determine the dry biomass weight.

Chlorophyll *a* was determined spectrophotometrically on day eight after extraction with 95 % ethanol for five minutes in a water bath at 70 °C (Lichtenthaler, 1987) and subsequent refrigeration at 4 °C for twenty-four hours under dark conditions for a maximum chlorophyll extraction. The absorbance was measured at 664 and 649 nm through spectrophotometer (U-1900, Hitachi) against prepared blank and the chlorophyll *a* concentration was computed following Lichtenthaler and Buschmann (2001).

2.7. Productivity of A. platensis

Productivity of *A. platensis* was calculated according to Danesi *et al*. (2011) using the following equation:

$$P_X = (X_m - X_i)(T_c)^{-1} \quad (1)$$

where,

P_X = productivity (g L^{-1} day^{-1}),

X_i = initial biomass concentration (g L^{-1}),

X_m = maximum biomass concentration (g L^{-1}), and

T_c = cultivation duration related to X_m (days).

2.8. Specific Growth Rate of A. platensis

Specific growth rate of *A. platensis* was calculated according to Madkour *et al*. (2012) using the following equation:

$$\mu_m = (\ln X_m - \ln X_i)(T_c)^{-1} \quad (2)$$

where,

μ_m = maximum specific growth rate (µ d^{-1})

X_i = initial biomass concentration (g L^{-1}),

X_m = maximum biomass concentration (g L^{-1}), and

T_c = cultivation duration related to X_m (days).

2.9. Data analysis

The maximum biomass dry weight, chlorophyll *a* content, productivity and specific growth rate of *A. platensis* cultured in control and the two treatments (T1 and T2) were analyzed using SPSS software (version 21) by the one-way independent analysis of variance (ANOVA) followed by Tukey HSD (Honestly Significant Difference) multiple comparison test.

3. Results

The average light intensity and air temperature throughout the cultivation period are shown in Figure 1. Light intensity and atmospheric temperature were relatively higher in non-shaded outdoor (field) conditions compared to shaded outdoor (greenhouse) and indoor (laboratory) conditions during daytime. Under the controlled laboratory conditions, light intensity and air temperature were stable at 24.66 ± 0.308 µmol m^{-2} s^{-1} and 27.5 ± 0.44 °C respectively throughout the day. Besides, the solar radiation under shaded outdoor conditions varied from 1.2 µmol m^{-2} s^{-1} to 367.0 µmol m^{-2} s^{-1} throughout the day. The average atmospheric temperature in this conditions varied from 24.7 °C - 28.7 °C in the morning, 31.5 °C - 34.6 °C during midday and 31.3 °C - 32.1 °C in the late afternoon. Meanwhile, the average light intensity in non-shaded outdoor conditions fluctuated between 4.2 µmol m^{-2} s^{-1} - 413.8 µmol m^{-2} s^{-1} in the morning, 1,007.0 µmol m^{-2} s^{-1} - 1,311.7 µmol m^{-2} s^{-1} during midday, and

27.2 µmol m^{-2} s^{-1} - 500.3 µmol m^{-2} s^{-1} in the late afternoon. The average ambient temperature in this conditions fluctuated between 25.5 °C - 29.9 °C in the morning, 35.8 °C - 39.9 °C during midday, and 32.8 °C - 35.6 °C in the late afternoon.

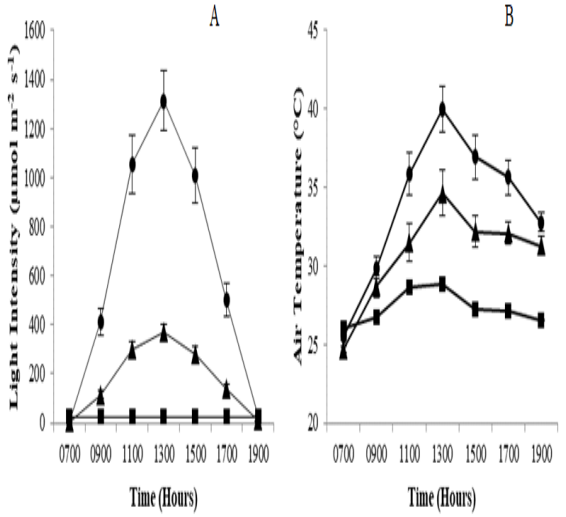

Figure 1. Average light intensity (A) and air temperature (B) throughout the cultivation duration in the laboratory; (■), shaded outdoor in greenhouse; (▲) and non-shaded outdoor in field; (●). Values are presented as mean ± SE (n = 8)

The result of growth curves based on optical density and biomass dry weight of the *A. platensis* culture under three culture conditions were illustrated in Figure 2. Based on the plotted growth curves, there was no presence of lag phase due to the preadaptation process. The growth curves also show incredible growth of *A. platensis* under non-shaded outdoor conditions compared to shaded outdoor and indoor controlled conditions.

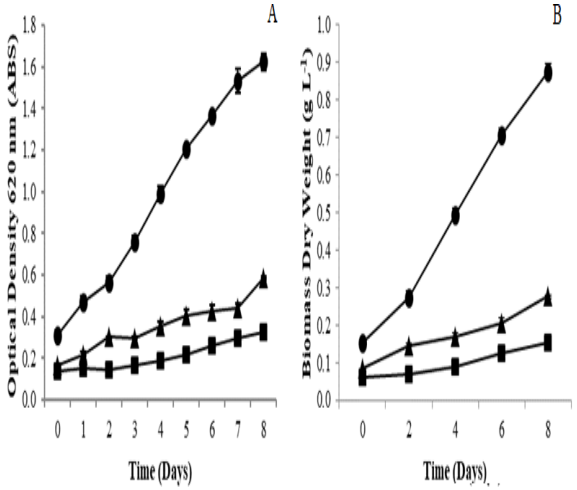

Figure 2. Optical density (A) and biomass dry weight (B) of *A. platensis* grown in different cultivation conditions. Control in laboratory; (■), T1 in shaded outdoor in greenhouse; (▲), T2 in non-shaded outdoor in field; (●). Values are presented as mean ± SE (n = 5)

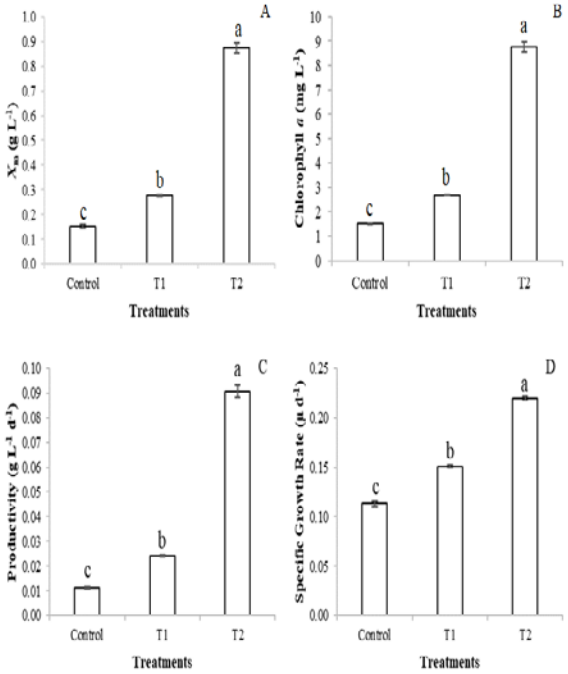

Figure 3. Maximum biomass dry weight, X_m (A), chlorophyll *a* (B), productivity (C) and specific growth rate (D) of *A. platensis* grown in different cultivation conditions. Control; (laboratory), T1; (shaded outdoor in greenhouse), T2; (non-shaded outdoor in field). Values are presented as mean ± SE (n = 5). Means with different letters (a-c) differ significantly ($p < 0.05$)

Figure 3 shows the maximum yield of *A. platensis* on day eight in terms of: biomass dry weight, chlorophyll *a* content, productivity and specific growth rate. This blue-green alga produced significantly higher ($p < 0.05$) biomass dry weight, chlorophyll *a* content, productivity and specific growth rate under non-shaded outdoor conditions (T2) with 0.88 ± 0.020 g L^{-1}, 8.77 ± 0.219 mg L^{-1}, 0.091 ± 0.0022 g L^{-1} d^{-1} and 0.220 ± 0.0017 µ d^{-1} respectively, followed by shaded outdoor conditions (T1) with 0.28 ± 0.005 g L^{-1}, 2.68 ± 0.022 mg L^{-1}, 0.024 ± 0.0004 g L^{-1} d^{-1} and 0.151 ± 0.0014 µ d^{-1} respectively. Meanwhile, microalga cultured in control under laboratory conditions achieved significantly lower ($p < 0.05$) biomass dry weight (0.15 ± 0.007 g L^{-1}), chlorophyll *a* (1.52 ± 0.024 mg L^{-1}), productivity (0.011 ± 0.0005 g L^{-1} d^{-1}) and specific growth rate (0.113 ± 0.0027 µ d^{-1}).

4. Discussion

The growth and productivity of *A. platensis* were significantly higher ($p < 0.05$) under outdoor non-shaded conditions compared to laboratory and shaded outdoor conditions probably due to one or to a combination of factors. Meteorological factors particularly irradiance flux and temperature have highly influenced the growth of microalga under outdoor conditions as these two factors largely vary throughout the day as the consequences of diurnal effect, cloud cover, rainfall and sometimes haze. In the present investigation, the average light intensity in outdoor non-shaded conditions was about 3.6-fold higher than shaded outdoor conditions (between 0700 and 1900); about forty times higher than laboratory conditions (between 1100 and 1700). On average, the recorded light intensity in shaded outdoor conditions was about 11-fold

higher than the laboratory conditions (between 1100 and 1700). Meanwhile, on average, the temperature in shaded (greenhouse) and non-shaded outdoor (field) conditions was higher about 5 °C and 10 °C respectively than in laboratory conditions (between 1100 and 1700). Moreover, the average temperature was increased about 10 °C and 15 °C in shaded and non-shaded outdoor conditions respectively from 0700 to 1300.

Besides, the light intensity and temperature reached the maximum during midday, which exceeded 1,300 µmol m^{-2} s^{-1} and 40 °C respectively under outdoor non-shaded conditions. Previous studies conducted under outdoor conditions showed reduced growth rate, photosynthetic efficiency, and chlorophyll content during such peak hours mainly due to photoinhibition (Vonshak *et al*., 2014; Torzillo *et al*., 1998; Chanawongse *et al*., 1994). Photoinhibition is light-induced photo-oxidative stress occurring at light intensities above the saturation of the photosynthetic rate, which causes severe photo-damage to photosynthetic pigments and, in extreme cases, a total loss of the algal culture (Soletto *et al*., 2008; Jensen and Knutsen, 1993). Moreover, the culture temperature between 35 °C and 37 °C was found to be optimum for microalgal biomass productivity (Richmond, 1988) while, a further increase in temperature was observed to hinder the growth rate due to inactivation of PSII activity (Chaiklahan *et al*., 2007). Previous investigations conducted on outdoor cultivation with 12:12 photoperiod also emphasized the night biomass loss due to respiration during the dark cycle (Edmundson and Huesemann, 2015; Phang *et al*., 2000), which influences the net *Arthrospira* productivity.

Despite the former reports on the possible limitations and inhibitions associated with microalgal farming in the natural ecosystem, *A. platensis* cultivation under outdoor non-shaded conditions in the present investigation, produced satisfactory growth within a cultivation period of eight days. The maximum algal growth in outdoor non-shaded conditions was 2.8, 3.2, 3.3, 3.8 and 1.5-fold higher than in shaded outdoor conditions and 5.0, 5.7, 5.8, 8.0 and 1.9-fold higher than in laboratory conditions in terms of optical density, biomass dry weight, chlorophyll *a* content, productivity and specific growth rate respectively. The present result seems better than a recent study conducted by Fagiri *et al*. (2013) who cultured *A. platensis* strain UTEXLB2340 under outdoor Malaysian conditions using Zarrouk's medium with a maximum optical density of 1.04 ABS (at 560 nm) over a cultivation period of twenty days. The growth of this blue-green alga cultured in reduced amounts of nutrients (half concentration Kosaric medium) within eight days in the present study is considered slightly better compared to the previous investigations conducted under indoor and outdoor conditions in various regions with longer cultivation periods as shown in Table 1.

A number of cultivation techniques were implemented in the present study aiming to maximize the productivity of this cyanobacterium under outdoor cultivation by reducing the inhibitory effects of the fluctuating extreme environmental conditions. Initially, the *A. platensis* culture was pre-adapted to the outdoor conditions through repeated batch cultivation processes for about two months. Previous studies demonstrated that algal cells acclimatized to the changes in growth conditions were less susceptible to the stress factors associated with the new environment compared to the non-acclimatized culture (Grobbelaar, 2007; Vonshak *et al*., 1996). Such acclimation involves an increase in the respiratory activity and a partial retrieval of photosynthetic activity in the *Arthrospira* cells after the initial drop due to exposure to stress conditions (Vonshak *et al*., 1988). The absence of lag phase in the growth curves (Figure 2) of *A. platensis* cultured in different conditions showed the adapted stage of this microalga to the new environment (outdoor conditions). Next, inoculation of alga for the new cycle was done during sunset to encourage the initial culture growth rate throughout the night under cool and dark conditions as the new culture is less tolerant to high light and temperature occurring during midday under outdoor conditions due to low cell density (Vonshak *et al*., 2014).

Next, the culture was continuously aerated throughout the cultivation period. Agitation is an important operational factor to reduce the prolonged exposure of algal cells to photo-inhibition and photo-limitation (Richmond, 2004) while allowing for a better assimilation of nutrients by *A. platensis* and enhancing gas exchange (Ogbonda *et al*., 2007). Accordingly, Vonshak *et al.* (2014) noted significantly smaller reduction in the maximal photochemical efficiency of PSII at the higher turbulence compared to the cultures with the lower turbulent flow during midday. A similar result was observed in other former studies (Grobbelaar, 1994; Richmond, 1992). Besides, the evaporated culture medium in the present investigation due to the high temperature during midday was compensated by tap water in order to maintain the salinity and pH of the growth conditions. This was done to avoid the sudden salinity and alkalinity shock during the cultivation period, which could hinder the algal productivity (Zeng and Vonshak, 1998). On the whole, the aforementioned cultivation techniques can be applied in such a way to achieve superior growth and productivity of alga compared to those occurring under indoor controlled conditions and shaded outdoor conditions.

Table 1. Growth of *Arthrospira* in present study compared to previous investigations

Medium	X_m (g L^{-1})	P_X (g L^{-1} d^{-1})	μ_m (d^{-1})	Chl a_{max} (mg L^{-1})	Duration	Cultivation conditions	References
Zarrouk's	1.18	0.075	0.109	8.81	15 d	Laboratory (30 °C in 50 µmol photons m^{-2} s^{-1} with 14:10 h light:dark PP)	Kumari *et al*. (2015)
Zarrouk's	0.58	0.031	0.175	23.22	18 d	Laboratory (30±1 °C in 50 µmol photons m^{-2} s^{-1} with 12:12 h light:dark PP)	Raoof *et al*. (2006)
Zarrouk's	0.72	0.030	0.074	-	450 h	Greenhouse (30 °C with 12:12 h light:dark PP)	Colla *et al*. (2007)
50% Zarrouk's	0.99	0.044	0.106	5.71	20 d	Greenhouse (Transparent jars)	Goksan *et al*. (2007)
20% Zarrouk's	0.90	0.059	-	-	18 d	Greenhouse with forced aeration (25.3°C under 12:12 h light:dark PP)	Walter *et al*. (2011)
20% Zarrouk's	1.33	0.054	0.160	-	≈ 25 d	Greenhouse (open bioreactors)	Andrade and Costa (2008)
SOT + underground water	0.80	0.048	0.187	5.12	16 d	Greenhouse (semi-outdoor)	Kim *et al*. (2007)
SOT	1.05	0.042	-	-	20 d	Outdoor	Toyoshima *et al*. (2015)
50% Zarrouk's	1.20	0.079	0.177	-	15 d	Outdoor (Transparent jars)	Singhal and Kumar (2017)
50% SKM	0.88	0.091	0.220	8.77	8 d	Outdoor	Present study

X_m: Maximum biomass dry weight; P_X: Productivity; μ_m: Maximum specific growth rate; Chl a_{max}: Maximum chlorophyll *a*; d: Days; h: Hours; PP: Photoperiod

5. Conclusion

A. platensis cultivation under outdoor non-shaded conditions (field) gave superior results compared to indoor laboratory and outdoor shaded conditions (greenhouse). Previously, it was assumed that Malaysia has unfavorable cultivation conditions for the microalgal growth due to the frequent cloud covering and precipitation. However, in the present investigation, Malaysian climatic conditions were proven to support a maximum yield of *A. platensis* with appropriate cultivation methods such as pre-adaptation of the algal culture, inoculation at late evening, continuous agitation and compensation of the evaporated culture medium. Unlike the controlled laboratory conditions, microalgal production in the real environment using the available resources potentially reduced the production cost as it requires less energy and supervision. Integration of the algal production system into natural conditions, as per being practiced in the agricultural sectors for the crop farming, paves the way towards the practical application of algal farming in Malaysia for commercial purposes. On the other hand, this study also highlighted the significance of the acclimatization process in the optimization of algal growth and productivity, especially when introducing the microalgal cultures to the new cultivation conditions through avoiding the presence of the lag phase and a shorter culture period. Besides, the utilization of half concentration growth medium (SKM) with satisfactory algal growth also reduced beneficially the cost of algal production by discounting the nutrient cost. On the whole, the present study proved that the cultivation of *A. platensis* in outdoor conditions under Malaysian tropical climate through proper cultivation methods is technically and economically viable.

Acknowledgment

This project was supported by the Research University Grant Scheme (RUGS): vote number 9373900, from Universiti Putra Malaysia.

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JJBS

Jordan Journal of Biological Sciences

Volume 11, Number 4, September 2018
ISSN 1995-6673
Pages 427 - 434

A Comparative Study of Fish Assemblage and Diversity Indices in two Different Aquatic Habitats in Bangladesh: Lakhandaha Wetland and Atari River

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Received February 8, 2018; Revised March 14, 2018; Accepted March 24, 2018

Abstract

This study on fish assemblage and diversity indices in two different aquatic habitats namely Lakhandaha wetland and Atari River was carried out from June 2015 to November 2015. A total of sixty-two species which comprise ten orders and twenty-one families were recorded where thirty-eight of the species belong to the Lakhandaha wetland (LW) and fifty species were from Atari River (AR). The best-represented family in both habitats of the LW and AR was Cyprinidae comprising 42.11 % and 40.00 % of the total fish species, respectively. Multivariate analyses (ANOSIM and MDS) showed a significant difference (ANOSIM, $P < 0.002$, R = 0.99) in the species numbers between the two habitats. In case of similarity percentages analysis, the overall average dissimilarity of the two habitats was 63.38 %; eleven species were found responsible for this dissimilarity. The mean Simpson dominance index, the Shannon-Wiener diversity index, Pielou's evenness index and Margalef's richness index in the LW and AR habitats were counted as 0.91 and 0.94, 2.77 and 3.12, 0.67 and 0.66, and 4.83 and 5.87, respectively. Based on Shannon-Wiener diversity index, the Mann-Whitney U test showed a significant difference between the two habitats (U = 2.500, $P = 0.012$) and confirmed that the AR habitat was more diverse in the fish population than the LW habitat.

Keywords: Fish diversity, Diversity indices, Lentic and lotic habitat, Lakhandaha wetland, Atari River.

1. Introduction

The biological organization of a community is characterized by species diversity. Species diversity of a specific ecosystem indicates a stable and good environmental condition. Therefore, it is necessary to gather information on the structure of fish assemblage in order to provide an effective management and conservative plans for fisheries ecosystems (Fischer and Quist, 2014). Bangladesh is considered a low-lying riverine country due to the presence of plenty of rivers. Large numbers of big rivers together with the network of their tributaries and branches crisscross the country. The total length of rivers with their tributaries is about 24, 14 km and the area is of about 8, 53, 863 ha in the country (DoF, 2014). Adjacent to these river channels, many low-lying wetlands exist and their inundation during the monsoon season makes it home to hundreds of species of fish, plants, birds, and other wildlife (Alam and Hossain, 2012). Most of the aquatic species especially the fish and prawn enter in the inundated areas of the wetland from the adjoining rivers and canals to feed, grow during the monsoon months, and have the benefits of protection and improved water quality. Therefore, wetlands can be thought of as "biological supermarkets" where they provide large volumes of food and as a result a large number of fish species become attracted to this ecosystem. Fishes also use wetlands for completing their life-cycle. Among the various factors that influence the wetland ecosystem are depth, nature of catchment areas or river basin, and precipitation and duration of the connection to the river (Sugunan *et al*., 2000).Therefore, fish assemblage and diversity status sometimes differ between rivers and wetland habitats. Biodiversity is often astonishingly altered or overused to define the population of a community. It is a measure of the numbers of species that make up a biologic community and is considered as one of the most important aspects of community organization or structure. Several studies have been done on the biodiversity status in rivers and wetlands individually in Bangladesh (Joadder *et al*., 2016; Flura *et al*., 2015; Parvez *et al*., 2017; Rahman *et al*., 2015). However, only few studies compared the fish assemblage and diversity status of these two different habitats together. Generally speaking, such types of studies are completely lacking in Bangladesh. Therefore, being encouraged by this fact, the present study was designed to compare the fish assemblage

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and diversity status in the Lakhandaha Wetland and Atari River on the basis that they constitute models for lentic and lotic aquatic habitats.

2. Materials and Methods

2.1. Study Area and Duration

The study was conducted in the Atari River (AR) and Lakhandaha Wetland (LW), located in the Noagaon district of Bangladesh. The AR habitat is situated between 24.32^0 to 24.42^0 North latitude and 88.50^0 to 89.06^0 East longitudes flowing through the north-western part of Bangladesh. On the other hand, the LW habitat is situated on the western side of the AR habitat (Figure 1). The area of the LW habitat is 50 ha in the rainy season and 3.50 ha in the dry season.

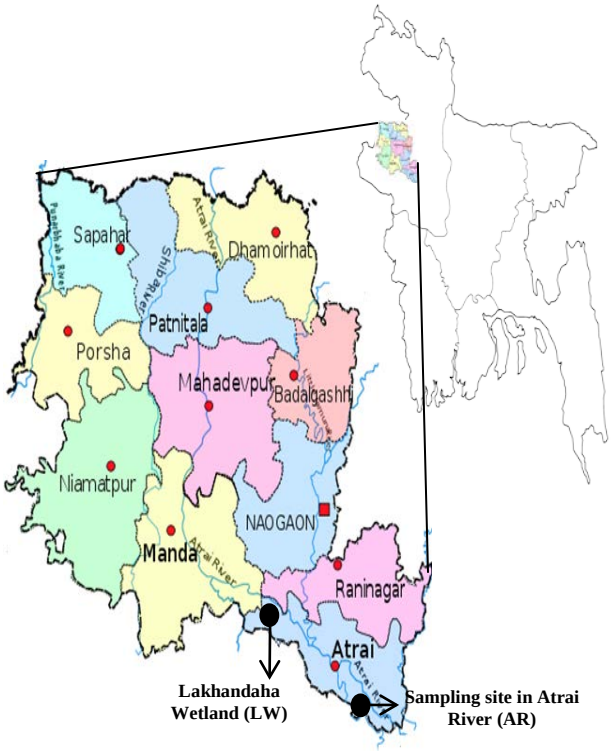

Figure 1. Location of the Lakhandaha wetland (LW) and Atari River (AR) habitats (sampling locations are shown by black dots).

2.2. Sampling Methods

Sampling was done over a period of six months from June 2015 to November 2015. The samples were taken on a monthly basis, *i.e* once per month. The collection of the fish samples from each habitat was done between 9 am to 5 pm. Three distinct places were sampled for each study site in each month. Traditional fishing nets namely cast nets, gill nets, and lift nets were used for the collection of the fish species. After harvesting, the counting of the fish species was done on the spot. However, the species that seemed difficult to identify on spot were preserved in 10 % buffered formalin solution and were transported to the laboratory of the Department of Fisheries at the University of Rajshahi, Bangladesh for identification and further study. These species were identified after analyzing their morphometric and meristic characters. Fish identification was done in accordance with Bhuiyan (1964), Rahman (1989, 2005) and Talwar and Jhingran (1991). The systematic classification of the identified fishes was done according to Nelson (2006).

2.3. Fish Diversity Analysis

The fish assemblage structure was estimated for each type of the habitat and it included: total specimens (N), Simpson index (1-D), Shannon diversity index (H'), Pielou's evenness index (J) and Margalef's species richness (S).

Simpson index is based on the following formula:

$$1 - D = 1 - \sum i \left(\frac{n_i}{N}\right)^2 \quad \text{(Simpson, 1949)}$$

Where n_i = is the number of individuals of taxon *I*, and N= is the total number of individuals. The Shannon diversity index is based on the formula:

$$H = -\sum i \frac{n_i}{N} \ln \frac{n_i}{N} \quad \text{(Shannon and Wiener, 1949)}$$

Where H= the diversity index, n_i = the relative abundance (S/N), *S*= the number of individual for each species, and *N*=total number of individuals.

Evenness index (J) is based on the following formula:

$$J = \frac{H}{L_{n^S[L_n = \text{The natural logarithm}]}} \quad \text{(Pielou's, 1966)}$$

Where H = is the Shannon-Wiener's diversity index, and S = is the number of different species in the sample.

Species richness (S) is based on the following formula:

$$D = \frac{S - 1}{\ln N} \quad \text{(Margalef, 1968)}$$

Where D = Margarlef's richness index, S = Number of different species in the sample, N = Total number of individual species in the sample.

2.4. Statistical Analysis

To represent the similarity among fish species assemblage based on presence/absence of data, the non-metric multi-dimensional scaling (MDS) was used. Similarity percentage analyses (SIMPER) (Clarke and Warwick, 1994) were also performed to observe the percentage contribution and average dissimilarity between the habitats. Similarity matrices were calculated using the Bray-Curtis similarity index (Bray and Curtis, 1957). A classical cluster analysis was run to examine the similarity among the fish assemblage in terms of $\log_{10}$ (x+1) transform data of fish abundance. Finally, Mann-Whitney U test (Brower *et al*., 1990) was performed to detect the differences in the fish diversity indices between the two habitats. Multivariate analyses were conducted using the software PAST 3 (Paleontological Statistics). For the statistical analysis, computer software SPSS (version 20) was used.

3. Results

3.1. Checklist of Fish Species Recorded

A total of 4018 fish were collected from both habitats (1252 from LW habitat and 2829 from AR habitat)

corresponding to twenty-one families and sixty-two species (thirty-eight in the LW and fifty in the AR habitat). The most-represented order in respect of the species number and composition percentage was: Cypriniformes, Siluriformes, Perciformes, and Channiformes in both habitats. In the LW habitat, order such as Beloniformes and Clupeiformes had no species recorded, and the orders Cyprinodontiformes and Tetraodontiformes comprised 1 % each of the total species. The contribution of Osteoglossiformes and Synbranchiformes constituted 2 % and 3 %, respectively of the total fish species found. On the other hand, in the AR habitat, Beloniformes, Clupeiformes, Cyprinodontiformes and Osteoglossiformes comprised 1 % each of the total species number whereas Synbranchiformes constituted 3 %. There were no species recorded for the order Tetraodontiformes from this habitat (Figure 2). However, in terms of families, the most representative were: Cyprinidae and Channidae in the LW habitat and Cyprinidae and Bagridae in the AR habitat. No species was recorded from the families Belonidae, Clupeidae, Pangasidae and Schilbeidae in the LW habitat, and the families Rasborinae, Cichlidae, Anabantidae, Heteropneustidae and Tetraodontidae in the AR habitat (Figure 3). Numerically dominant species included *Chanda nama* (14.78 %), *Esomous danricus* (13.18 %), *Parambassis ranga* (11.90 %), and *Trichogaster fasciatus* (6.07 %) in the LW habitat, while the dominant species in the AR habitat were *Amblypharyngodon mola* (8.84 %), *Gudusia chapra* (8.45 %), *Aspidoparia moror* (6.08 %), and *Chela laubuca* (5.51 %).

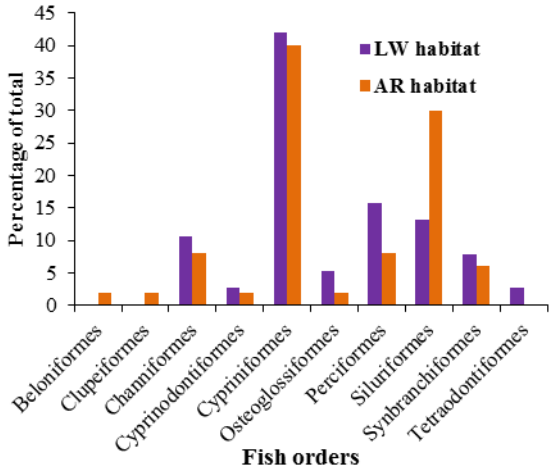

Figure 2. Comparison of order-wise percentage composition of fishes from Lakhandaha wetland (LW) and Atari River (AR) habitats.

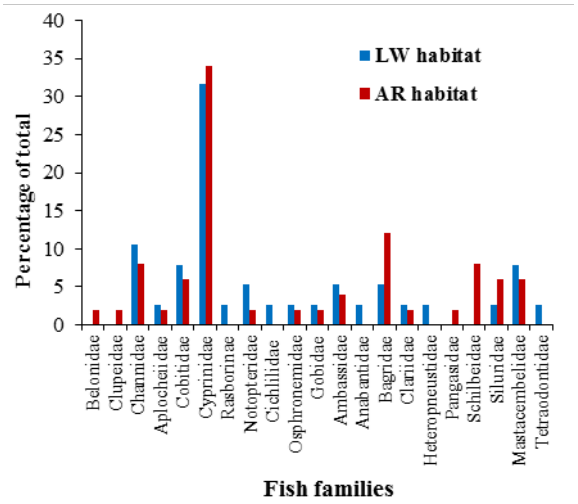

Figure 3. Comparison of family-wise percentage composition of fishes from Lakhandaha wetland (LW) and Atari River (AR) habitats.

3.2. Fish Assemblage

Fish assemblage composition of the two habitats was significantly different (ANOSIM, $P < 0.002$, R = 0.99; Figure 4). SIMPER analysis revealed the average percentage of dissimilarity of the species between the two different habitats. The overall average dissimilarity of the two habitats was 63.38 %. However, considering the lowest average contribution of each species at 2.66 %, eleven species were found to contribute most to this dissimilarity. These species are: *Eutropiichthys vacha*, *Cirrhinus reba*, *Ailia coilia*, *T. fasciatus*, *Pethia ticto*, *G. chapra*, *A. moror*, *Sperata aor*, *A. mola*, *Rita rita* and *E. danricus*. The cluster analysis classifies the whole fish species from the two habitats into three distinct categories (cluster A, B and C) at 42 % similarity (Figure 5). Cluster "A" represents twenty-two species of which ten were absent in the AR habitat. The other twelve species were found in this habitat but with a lesser number compared to the LW habitat. There were twenty-four species comprising cluster "B", and all of these species were absent in the LW habitat. However, the common species for both the LW and AR habitats are represented by cluster "C".

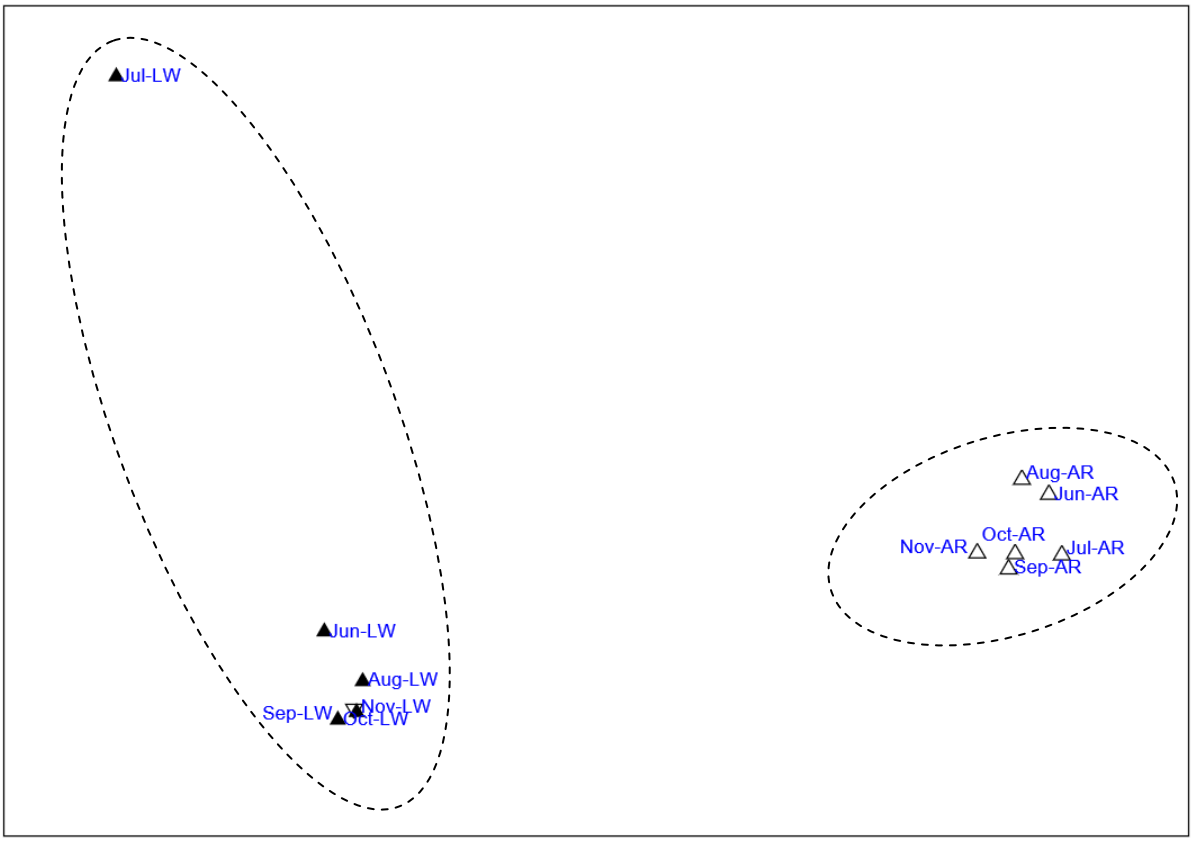

Figure 4. Non-multi-dimensional scaling (MDS) ordination depicting similarity/dissimilarity of fish assemblages from Lakhandaha wetland (LW) (fill triangles) and Atari River (AR) habitats (open triangles). Each symbol represents one sampling month. Relative distance among symbols represents the relative similarity/dissimilarity of assemblage composition from the site based on presence/absence of data.

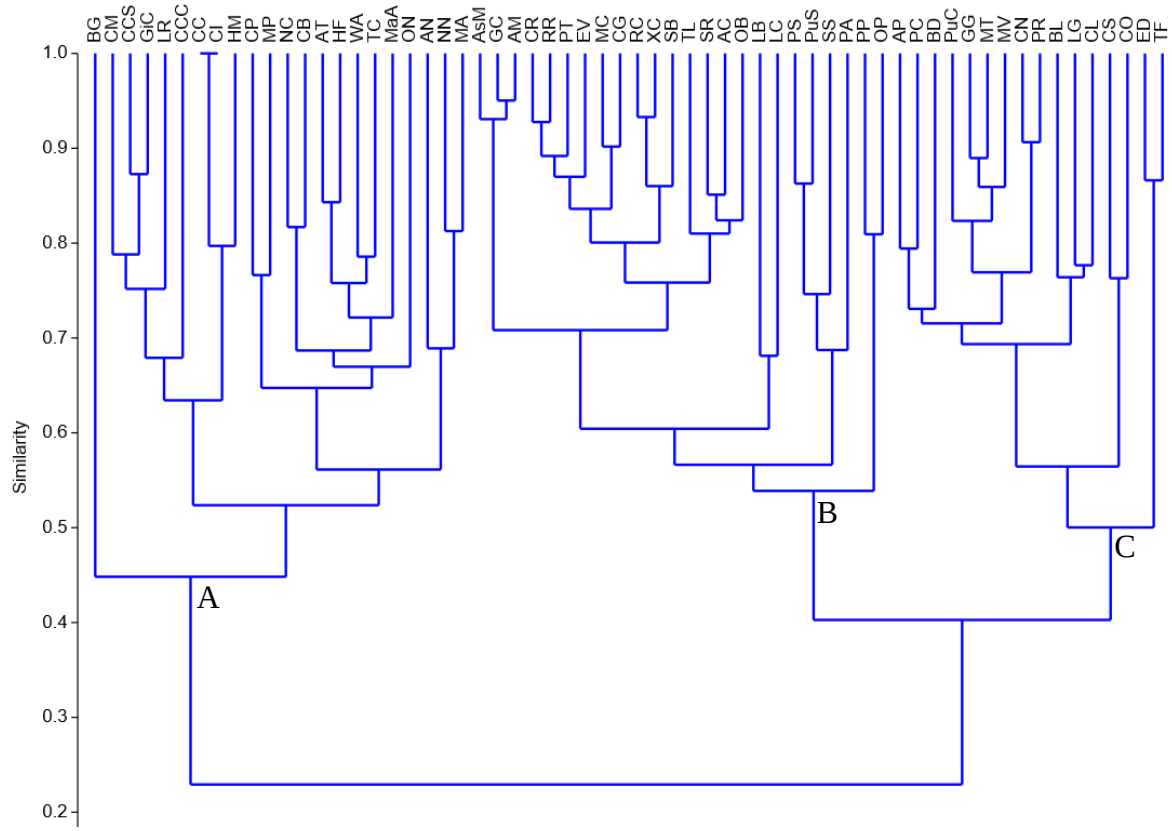

Figure 5. Dendrogram of the species assemblage for the two habitats using Bray-Curtis similarity measure. Data consist of $\log_{10}$ (x+1) transformed species abundance in numbers. Species code is given in Table 1.

Table 1. Checklist of fish species recorded from Lakhandaha wetland (LW) and Atari River (AR) habitats with their conservation status.

Order	Family	Scientific name	Species code	English name	Local name	LW habitat	AR habitat
Beloniformes	Belonidae	*Xentodon cancila* (Hamilton, 1822)	XC	Freshwater garfish	Kakila	A	P
Clupeiformes	Clupeidae	*Gudusia chapra* (Hamilton, 1822)	GC	Indian river shad	Chapila	A	P
Channiformess	Channidae	*Channa Striata* (Bloch, 1793)	CS	Snakehead murrel	Shol	P	P
		Channa punctatus (Bloch, 1793)	CP	Spotted snakehead	Taki	P	P
		Channa orientalis (Bloch and Schneider, 1801)	CO	Walking snakehead	Cheng	P	P
		Channa marulius (Hamilton, 1822)	CM	Great snakehead	Gozar	P	P
Cyprinodontiformes	Aplocheiidae	*Aplocheilus panchax* (Hamilton, 1822)	AP	Blue panchax	Pach chok	P	P
Cypriniformes	Cobitidae	*Botia dario* (Hamilton, 1822)	BD	Bengal loach	Rani, Bou	P	P
		Botia lohachata (Chaudhuri, 1912)	BL	Reticulate loach	Rani, Bou	P	P
		Lepidocephalus guntea (Hamilton, 1822)	LG	Guntea loach	Gutum	P	P
	Cyprinidae	*Amblypharyngodon mola* (Hamilton, 1822)	AM	Mola carplet	Mola	A	P
		Aristichthys nobilis (Richardson,1845)	AN	Bighead carp	Bighead	P	P
		Aspidoparia moror (Hamilton, 1822)	AsM	Aspidoparia	Morari	A	P
		Barbonymus gonionotus (Bleeker,1849)	BG	Silver barb, Java barb	Thai sarputi	P	P
		Chela laubuca (Hamilton, 1822)	CL	Indian GlassBarb	Laubuca/mulungi chela/ Chap chela	P	P
		Cirrhinus cirrhosis (Hamilton, 1822)	CC	Mrigal carp	Mrigal	P	A
		Cirrhinus reba (Hamilton, 1822)	CR	Reba	Raek	A	P
		Ctenopharyngodon idella (Hamilton, 1822)	CI	Grass carp	Grass carp	P	A
		Cyprinus carpio var. specularis (Hamilton, 1822)	CCS	Mirror carp	Mirror carp	P	A
		Cypricus carpio var. communis (Hamilton, 1822)	CCC	Common carp	Common carp	P	P
		Gibelion catla (Hamilton, 1822)	GiC	Indian major carp	Catla	P	A
		Hypophthalmiththys molitrix (Hamilton, 1822)	HM	Silver carp	Silver carp	P	A
		Labeo bata (Hamilton, 1822)	LB	Bata	Bata	A	P
		Labeo calbasu (Hamilton, 1822)	LC	Orange-fin labeo	Kalibaus	A	P
		Labeo rohita (Hamilton, 1822)	LR	Rohu	Rui	P	P
		Pethia conconius(Hamilton, 1822)	PC	Rosy barb, Red barb	Kachon punti	P	P
		Pethia ticto (Hamilton, 1822)	PT	Ticto barb	Tit punti	A	P
		Puntius chola (Hamilton, 1822)	PuC	Swamp barb	Chola puti	P	P
		Puntius sarana (Hamilton, 1822)	PS	Olive barb	Sarpunti	A	P
		Puntius sophore (Hamilton, 1822)	PuS	Pool barb	Jatpunti	A	P
		Rohtee cotio (Hamilton, 1822)	RC	Cotio	Keti (fish)	A	P
		Salmostoma bachila (Hamilton, 1822)	SB	Large razor belly minnow	Chela	A	P
	Rasborinae	*Esomous danricus* (Hamilton, 1822)	ED	Flying barb	Darkina, Darka	P	A
Osteoglossiformes	Notopteridae	*Notopterus chitala* (Hamilton, 1822)	NC	Clown knifefish	Chitol	P	A
		Notopterus notopterus (Hamilton, 1822)	NN	Bronze feather back	Foli	P	P
Perciformes	Cichlidae	*Oreachromis niloticus* (Hamilton, 1822)	ON	Nile tilapia	Nilotica	P	A
	Osphronemidae	*Trichogaster fasciatus* (Bloch and Schneider, 1801)	TF	Banded gourami	Boro kholisha	P	A
		Trichogater lalius(Hamilton, 1822)	TL	Dwarf gourami	Lal kholisa	A	P
	Gobidae	*Glossogobius giuris* (Hamilton, 1822)	GG	Tank goby	Bele	P	P
	Ambassidae	*Chanda nama* (Hamilton, 1822)	CN	Elongate glass-perchlet	NamaChanda	P	P
		Parambassis ranga (Hamilton, 1822)	PR	Indian glassy fish	Rangachanda	P	P
	Anabantidae	*Anabas testudineus* (Hamilton, 1822)	AT	Climbing perch	Koi	P	A

Order	Family	Scientific name	Species code	English name	Local name	LW habitat	AR habitat
Siluriformes	Bagridae	*Mystus cavasius* (Hamilton, 1822)	MC	Gangetic mystus	Gulsa tengra	A	P
		Mystus tengra (Hamilton, 1822)	MT	Bagrid catfish	Choto tengra	P	P
		Mystus vittatus (Hamilton, 1822)	MV	Striped dwarf catfish	Tengra	P	P
		Rita rita (Hamilton, 1822)	RR	Rita	Rita	A	P
		Sperata aor (Hamilton, 1822)	SA	Long-whiskered catfish	Air	A	P
		Sperata seenghala (Sykes, 1839)	SS	Giant-river catfish	Guizza air	A	P
	Clariidae	*Clarias batrachus* (Hamilton, 1822)	CB	Walking catfish	Magur	P	P
	Heteropneustidae	*Heteropeneustes fossilis* (Hamilton, 1822)	HF	Stinging catfish	Shing, Kanos	P	A
	Pangasidae	*Pangasius pangasius* (Hamilton, 1822)	PP	Pungas	Pangas	A	P
	Schilbeidae	*Ailia coilia* (Hamilton, 1822)	AC	Gangetic ailia	Kajuli	A	P
		Pseudeutropius atherionoides (Hamilton, 1822)	PA	Indian potasi	Batashi	A	P
		Clupisoma garua (Hamilton, 1822)	CG	Gaura bachcha	Ghaura	A	P
		Eutropiichthys vacha (Hamilton, 1822)	EV	Batchwa bacha	Bacha	A	P
	Siluridae	*Wallago attu* (Hamilton, 1822)	WA	Freshwater shark	Boal	P	P
		Ompok pabda (Hamilton, 1822)	OP	Pabdah catfish	Modhu pabda	A	P
		Ompok bimaculatus (Bloch, 1794)	OB	Butter catfish	Boili pabda	A	P
Synbranchiformes	Mastacembelidae	*Mastacembelus pancalus* (Hamilton, 1822)	MP	Barred spiny eel	Guchi	P	P
		Mastacembelus armatus (Hamilton, 1822)	MA	Zig-zag eel	Baim	P	P
		Macrognathus aculeatus (Bloch, 1783)	MaA	Lesser spiny eel	Tara baim	P	P
Tetraodontiformes	Tetraodontidae	*Tetradon cutcutia* (Hamilton, 1822)	TC	Ocellated pufferfish	Potka	P	A

Key: P = Present, A = Absent.

3.3. Diversity, Evenness and Richness Indices

Diversity, evenness and richness indices were calculated for 1252 and 2829 individuals from the LW and AR habitats, respectively. Mann-Whitney U test was applied to find out differences in indices between the two habitats. The Simpson dominance index was 0.91±0.03 in the LW habitat and 0.94±0.01 in the AR habitat. The Mann-Whitney U test showed a significant difference between the two habitats (U = 2.500, P = 0.012), with the AR habitat being more diverse than the LW habitat (Figure 6). Similar to the Simpson dominance index, Shannon-Wiener diversity index also showed significant difference between the two habitats (U = 4.00, P = 0.025) , where the highest value (3.12±0.19) was found for the AR habitat and the lowest (2.77±0.35) for the LW habitat. However, species evenness (U = 16.00, P = 0.748) and richness (U = 8.00, P = 0.109) index showed no significant difference between the habitats at all.

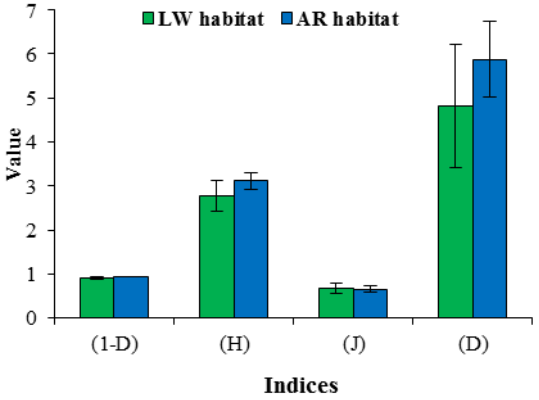

Figure 6. Values of species diversity, evenness and richness indices of the Lakhandaha wetland (LW) and Atari River (AR) habitats. (1-D), (H), (J) and (D) indicate Simpson index, Shannon-Wiener diversity index, Pielou's evenness index and Margalef's richness index, respectively.

4. Discussion

Comparing the fish biodiversity in the two different habitats, twenty-six species were found common to both habitat types, whereas, among the sixty-two species identified, twenty-four and twelve species were distinct in the LW and AR habitats. Among the taxa living in the lentic habitat, six species (*Cirrhinus cirrhosis*, *Ctenopharyngodon idella*, *Cyprinus carpio* var. *specularies*, *Gibelion catla*, *Hypopthalmichthys molitrix* and *Oreochromis niloticus*) were commercially produced in nearby fish ponds. Therefore, their presence in the LW habitat might be due to the over flooding of the culture pond in addition to some stocking practices of the local people. The remaining species were not commercially produced, and are thus native species that are somehow resistant to pollution. The number of the fish species recorded during the study period was higher in the AR habitat (fifty species) compared to the LW habitat (thirty-eight species). The number of fish species recorded from the AR habitat was more or less similar to the results obtained by Parvez *et al*. (2017) in Dhepa river (fifty-five species), Rahman *et al*. (2015) in Talma River (fifty-six species) and Mohsin *et al*. (2014) in Andharmanik River (fifty-three species). However, the species number was much lower than the findings of Galib (2015) in Brahmaputra River (sixty-seven species) and Joadder *et al*. (2015) in Padma River (seventy-one species). In the LW habitat, the number of species recorded was much lower than the findings of Sultana *et al*. (2017) in Bhawal beel (fifty-six species), Joadder *et al*. (2016) in Kumari beel (fifty-two species), Flura *et al*. (2015) in Balla beel (seventy-four species), Akhtaruzzaman and Alam (2014) in Ichanoi beel (sixty-two species), Imteazzaman and Galib (2013) in Halti beel (sixty-three species). During the study period, habitat loss, over-exploitation, and the indiscriminate killing of juvenile fish due to unregulated fishing pressures, the destruction of breeding and nursery grounds were observed which might be responsible for the less diversity of fish fauna in the studied wetland (LW habitat). Siddiq *et al*. (2013), Galib *et al*. (2009) and Chakraborty and Mirza (2007) detected more or less the same reasons behind the decline of fish diversity which supports the present findings. Based on the order, percentage analysis of the existing species of the fish showed the highest occurrence under the three orders namely Cypriniformes, Perciformes, and Siluriformes in the lentic habitat. While in the AR habitat, the three richest orders were Cypriniformes, Siluriformes, and both of Perciformes and Channiformes. The above-mentioned findings are usual because these three Orders (Cypriniformes, Siluriformes and Perciformes) are the most dominant groups in the freshwater bodies of Bangladesh (Rahman, 2005). The highest percentage of family composition in both the LW and AR habitats was Cyprinidae, which was previously reported by De *et al*. (2011) who mentioned that Cyprinidae represents a major contribution with a large number of species in different open water bodies of Bangladesh. Imteazzaman and Galib (2013), Siddiq *et al*. (2013), Joadder *et al*. (2016), Akhtaruzzaman and Alam (2014) also recorded Cyprinidae as the dominant family. The number of species was absent for the order Beloniformes and Clupeiformes in the LW habitat. Those fish species were *X. cancila* and *G. chapra* belonging to the family Belonidae and Clupeidae, respectively. However, only one species was obtained for these two orders in the AR habitat during the period of this investigation. Species such as *T. fasciatus* and *E. danricus* were reported to distinctively inhabit shallow waters. Therefore, they were most abundant in the LW habitat. On the other hand, *S. aor*, *A. mola*, *R. rita*, *E. vacha*, *C. reba*, *G. chapra* and *A. moror* were abundantly found in the AR habitat. Therefore, these were the species mostly responsible for the differentiation between the two habitat types. The researchers detected a high species richness (5.87) and diversity (3.12) index in the AR habitat, suggesting a healthy environment with little alterations. Therefore, during the present study, the fish species were not evenly distributed between the two habitats based on the diversity index value which was smaller than 4.6 as Bibi and Ali (2013) mentioned that the diversity index of less than 4.6 indicates an uneven distribution of avian communities at *Taunsa barrage* wildlife sanctuary. During the study period, Shannon-Wiener diversity index of both the LW (2.77) and AR (3.12) habitats was within the range reported by Iqbal *et al*. (2015) (2.90-3.12) in a freshwater haor of Bangladesh. However, Pielou's evenness index (0.67) was below and Margalef's richness index (4.83) was above the range (0.82-0.88 and 3.02-2.70) reported by the same author. Changes in the present biodiversity indices between the two habitats might be associated with the spatial, hydrological, and biological combination of the defined area.

5. Conclusion

In conclusion, the AR habitat was found more suitable for a large number of species with a wide range of fish communities. However, the suitability of the LW habitat was lost due to several destructive fishing practices. Therefore, conservation might be an essential plan for improving the fish species in this habitat. Knowledge gathered through the present study should be incorporated into decision-making processes for the conservation of the fish diversity in the open water bodies of Bangladesh. Although the loss of biodiversity is being an alarming threat at the present, early and timely effective managements are very essential to deal with this issue.

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JJBS

Jordan Journal of Biological Sciences

Volume 11, Number 4, September 2018
ISSN 1995-6673
Pages 435 - 439

Development and Validation of Conventional PCR for the Detection of the sctQ Gene

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Received January 30, 2018; Revised March 21, 2018; Accepted March 26, 2018

Abstract

Burkholderia pseudomallei can potentially cause lethal infections if not diagnosed properly. At present, several PCR assays have been developed for *B. pseudomallei* identification, with variable sensitivities and specificities. In this report, a PCR assay targeting a broad-range *B. pseudomallei* highly conserved gene of the type three secretion system-1 (TTS1) cluster was developed and was validated against a panel of multi-locus sequence types of clinical *B. pseudomallei* isolates. The analytical sensitivity and specificity were 100 % each. The lower limits of detection of purified DNA and bacterial cells spiked in blood specimens were 100 fg/µL and 18.4 x 10^5 CFU/mL, respectively. This study demonstrates the utility of the TTS1 gene for the *B. pseudomallei* identification.

Keywords: Melioidosis, *Burkholderia pseudomallei*, PCR, TTS1, MLST, sctQ gene

1. Introduction

Melioidosis is a disease of varying severity that affects both humans and animals, and that can be fatal if left untreated (Podnecky NL, *et al.*, 2013). It is caused by *Burkholderia pseudomallei* that can be readily recovered from the water and soil in endemic areas, such as Northern Australia and Southeast Asia (Puthucheary SD, 2009). The clinical presentation of melioidosis includes non-septicemic subclinical infection, cutaneous lesions, or severe septicemia that may disseminate to a single or multiple organs (Zueter *et al*., 2016a). In an acute infection state, death may occur within twenty-four to forty-eight hours of the onset of symptoms. The mortality rate can reach up to 40 %. In addition, *B. pseudomallei* is intrinsically resistant to many of the broad spectrum antibiotics (Haase *et al.*, 1998, Podnecky *et al.*, 2013). Delay in the isolation and identification of *B. pseudomallei* contributes to the high mortality rates in more than 50 % of the patients during the first two days after hospital admission and before obtaining positive bacterial culture results (Dharakul *et al.*, 1996).

At present, several PCR assays have been developed for *B. pseudomallei* identification, with variable sensitivities and specificities. It was suggested that a gene targeted by a PCR assay might not be present among all *B. pseudomallei* isolates, which may compromise detection performance (Novak *et al.*, 2006). The type three secretion system-1 (TTS1) gene cluster was reported as being universally present among *B. pseudomallei* (Winstanley and Hart, 2000, Novak *et al.*, 2006). In this study, a PCR assay targeting the highly conserved TTS1 cluster was developed and validated against a panel of MLST-strain types of *B. pseudomallei*.

2. Materials and Methods

2.1. Primers Design

In this study, a previously identified area located adjacent to the *orf1-stcQ* gap in the TTS1 gene cluster (GenBank accession code: AF074878) (Holden *et al.*, 2004) was targeted for PCR amplification based not only on its *B. pseudomallei* theoretical specificity, but also on its apparent ubiquitous distribution among clinical and environmental isolates (Smith-Vaughan *et al.*, 2003). Forward and reverse primers were constructed using Primer-Blast service www.ncbi.nlm.nih. gov/tools/primer-blast/. Forward and reverse primers target a 316-bp region located in putative gene *sctQ* (29 to 344) and on TTS1 (24015 to 24433): *sctQ*F (5`-CACACTTCAACG CGACACTG-3`) and *sctQ*R (5`-GGGAGCTCGATG ACATAGCC-3`). Primers were synthesized and provided as desalted lyophilized form from 1stBASE, Singapore.

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2.2. Collection and Preparation of Bacterial Strains

The study utilized thirty-eight archived and newly-recovered *B. pseudomallei* isolates from different clinical samples obtained from melioidosis patients admitted to Hospital Universiti Sains Malaysia, at Kelantan state from 2007 to 2014. Bacteria were reactivated by aerobic cultivation on Tryptone soya agar (Oxoid Ltd., Basingstoke, United Kingdom). Similar reactivation procedure was applied on archived non-*B. pseudomallei* bacteria that were obtained from the stock culture unit of Medical Microbiology and Parasitology Department at Universiti Sains Malaysia. The study was approved by the Research Ethics Committee (Human) (USM/JEPeM/ 15110495).

2.3. DNA Extraction

Dense bacterial suspensions were prepared in 10 mL Tryptone soya broth and were subjected to genomic DNA extraction using DNeasy tissue kit (Qiagen Inc., Hilden, Germany) according to the manufacturer instructions. In addition, DNA from other bacteria, fungi, and from human blood was prepared. For detection limit determination, 10-fold serial dilution of extracted *B. pseudomallei* DNA was made in elution buffer beginning from 1 ng/µL to 1 fg/µL. For spiked samples, 2 mL EDTA-blood was centrifuged at 1500 g for ten minutes, and 200 µL of the resultant buffy coat were subjected to DNA extraction using the QIAamp DNA blood mini kit (Qiagen Inc., Hilden, Germany) according to the manufacturer instructions. In both protocols, the final incubation in the elution step was extended to thirty minutes to increase the DNA yield.

2.4. PCR Assay

The optimized PCR mixture contained 0.5 µL of extracted DNA in 25 µL final reaction volume consisting of PCR mastermix containing 1x MyTaq Red reaction buffer (Bioline Ltd UK), comprised of 5 mM dNTPs, 15 mM $MgCl_2$, stabilizers and enhancers, and 1 U/µl MyTaq DNA polymerase (Bioline Ltd UK), a final concentration of 0.2 µM of each forward and reverse primers, and PCR water. Amplification was performed with Master Cycler Nexus Gradient (Eppendorf, Germany) using standard settings. The optimized thermal profile was initial denaturation at 95ºC for four minutes, followed by thirty cycles of 95ºC:15 s, 65ºC:15 s, and 72ºC:30 s, and a final extension at 72ºC:4 min. No-template (PCR water) and positive *B. pseudomallei* controls were included in each run to rule out amplification failure or possible contamination.

An internal control pET32a plasmid and its primers (T7 Promoter780-764 5' TAATACGACTCACTATAG 3' and T7 Terminator 168-185 5' GCTAGTTATTGCTCAGCGG 3'), provided by Novagen, USA, were added to rule out the possibility of amplification inhibition induced by clinical specimen constituents. Plasmid volume and the final concentrations of its primers were suited to run in duplex with the *sctQ*-PCR. Using 1.5 % agarose gel loaded with Gelred stain (Bioline Ltd UK), PCR products along with DNA ladder (10kb Hyperladder™I, Bioline Ltd, UK) electrophoresis was performed and the separated amplicons were visualized by ultraviolet light transilluminator and computerized image analysis system (G-Box, Syngene, USA).

2.5. PCR Assay Sensitivity

For sensitivity and specificity, the concentration of all tested DNA templates was normalized at 1 ng/µL using NanoDrop UV-Vis spectrophotometer (Thermo Scientific, Wilmington, DE). The genetic and geographic spectrum of detection of the sctQ-PCR assay was assessed using DNA from 83 *B. pseudomallei* isolates encompassing thirty-two different genotypes obtained from different distinct population communities of peninsular Malaysia. In addition, up to half of the isolates' genotypes were previously identified in other surrounding south-eastern countries, and most of them have suggested Australian ancestry. The isolates were provided by Zueter *et al* (2018).

2.6. PCR Specificity

Discriminative specificity of the *sctQ*-PCR assay was determined by screening against closely related species including *Burkholderia* spp. and *Pseudomonas* spp. We also evaluated the assay using clinically important non-*Burkholderia* bacteria and eukaryotic species (Table 1).

Table 1. Organisms* (*n=72*) used to test the specificity of the *sctQ*-PCR assay

Organism category	Name of organism	Count tested
Genetic relatives (n=27)	*Burkholderia cepacia KR869104*	2
	Burkholderia cepacia	9
	Burkholderia thailandensis KR869105 (ST77)	1
	Pseudomonas aeruginosa	7
	Pseudomonas lutelo,	1
	Pseudomonas stutzeri	1
	Pseudomonas flurosence	1
	Stenotrophomonas maltophilia	1
	Acinetobacter baumannii	1
	Chryseobacterium indologens	1
	Chromobacterium violaceum	1
	Moraxella catarrhalis	1
Other types of bacteria (n= 37)	*Shigella boydi,*	1
	Shigella dysentri,	1
	Shigella sonnie	1
	Shigella flexeri	1
	Salmonella enterica	1
	Salmonella typhi	1
	Salmonella paratyphi	1
	Proteus merabillis	1
	Proteus volgaris	1
	Escherichia coli	1
	Citrobacter freundi	1
	Klebsiella pneumoniae	1
	Enterobacter cloacae	1
	Staphylococcus aureus	2
	Streptococcus pneumoniae	2
	Streptococcus group A	1
	Streptococcus group B	1
	Streptococcus group G	1
	Streptococcus group D	1
	Listeria monocytogens	1

	Mycobacterium tuberculosis	3
	Mycobacterium bovis	1
	Haemophilus influenza	2
	Neisseria meningitides	1
	Liptospira interrogans	1
	Aeromonas hydrophila	1
	Helicobacter pylori	4
	Vibrio mimicus	1
	Vibrio cincinnatiensis	1
Other organisms (n=8)	Human blood cells	4
	Candida albicans	1
	Candida tropicalis	1
	Candida glabrata	1
	Cryptococcus neoformans	1

*Identification of isolates was confirmed by biochemical tests. MLST and 16S rRNA typing were done for *Burkholderia species*.

2.7. The Lower Limit of Detection

Ten-fold serial dilution of extracted *B. pseudomallei* DNA was made in elution buffer beginning from 1 ng/µL to 1 fg/µL. Optimized *sctQ*-PCR run along with positive and negative controls was done for every dilution in duplicate. The end products were subjected to electrophoresis followed by visualization.

The PCR assay performance was further assessed by determining the lowest detectable concentration of *B. pseudomallei* cells in spiked blood samples. In a class-II biosafety cabinet, *B. pseudomallei* cells' count of 10^9 colony-forming units per milliliter of sterile phosphate buffered saline (CFU/mL), was prepared via comparison to McFarland (McF) standard. Count was confirmed by the agar-dilution method. One milliliter of the suspension was transferred into 1.5 mL microtube. The suspension was then serially diluted up to a concentration of 1.0 CFU/mL. A 100 µL aliquot from each dilution was spread onto tryptone soya agar (TSA) plates, and was incubated for seventy-two hours at 37ºC. The rest of the volume (900 µL) of each individual dilution was used to spike ten 2.0 mL EDTA anti-coagulated blood samples.

For quality control, 100 µL of PBS was plated on TSA for seventy-two hours to confirm sterility. In addition, 1 mL of sterile PBS was added to 2.0 mL EDTA blood, was mixed thoroughly, and processed along with all spiked blood samples for DNA extraction and PCR. DNA was extracted from the buffy coat. The optimized *sctQ*-PCR was applied on DNA extracted from all spiked blood samples and controls. The internal control pET32a plasmid and its primers were added to all PCR runs, to insure the absence of the inhibition of amplification reactions.

3. Results

For *in-silico* specificity, DNA sequencing for the *sctQ*-PCR purified amplicons was done and showed complete match once aligned to a reference sequence. *sctQ*-PCR showed 100 % analytical sensitivity and specificity (Figure 1). No amplification for non-*B. pseudomallei* was observed, while observing amplification bands for the internal control which confirms the absence of amplification inhibitors, and validates the negative results for non-*B. pseudomallei* controls (Figure 2).

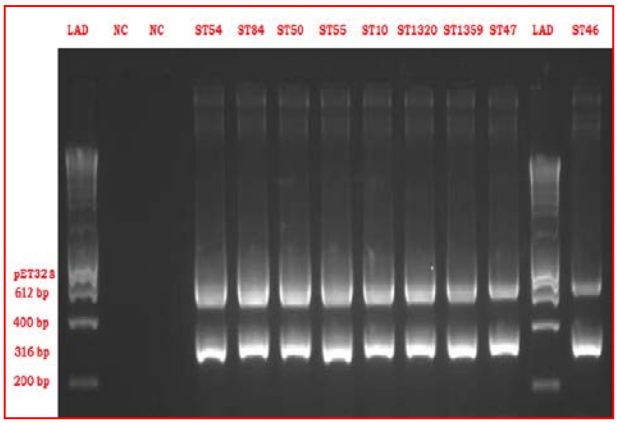

Figure 1. Gel electrophoresis of duplex PCR performed on different sequence types of *B. pseudomallei*. Gel showing amplicon bands for *sctQ* (316 bp) and for the internal control (pET328). LAD: 10kb DNA ladder; NC: negative control; ST: sequence types of *B. pseudomallei*.

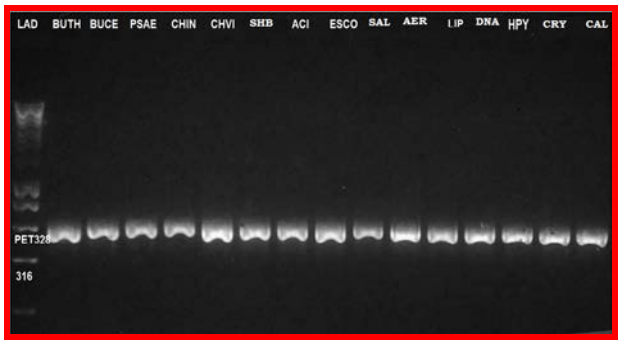

Figure 2. Duplex PCR of sctQ primers and plasmid pET328 internal control plasmid against non-B. pseudomallei isolates. LAD: 10kb DNA ladder; BUTH: Burkholderia thailandensis, BUCE: Burkholderia cepacia, PSAE: Pseudomonas aeruginosa, CHIN: Chryseobacterium indologenes, CHVI: Chromobacterium violaceum, SYB: Shigella boydii, DNA: human blood, ESCO: Escherichia coli, SAL: Salmonella enterica serovar enteritidis, AER: Aeromonas hydrophila, LIP: Leptospira interrogans, HAI: Haemophilus influenzae, HYP: Helicobacter pylori, CRY: Cryptococcus neoformans, CAL: Candida albicans.

The lower concentration of purified *B. pseudomallei* DNA that was amplified by *sctQ*-PCR was 100 fg/µL, whereas the lowest number of bacterial cells detected in spiked blood specimens was 18.2 X 10^5 CFU/mL (Figures 3 and 4).

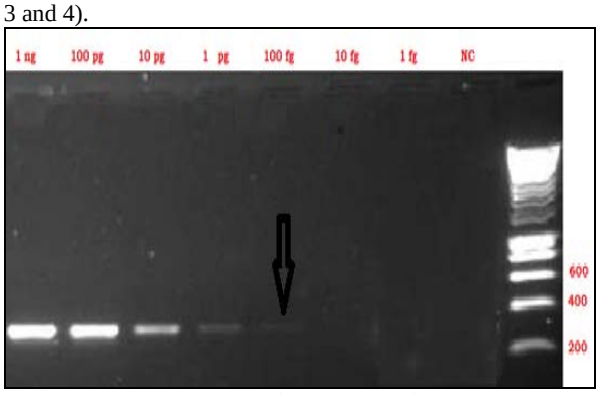

Figure 3. Gel electrophoresis of *sctQ*-PCR performed on gradient concentrations of purified *B. pseudomallei* DNA. The arrow indexes for the lower concentration of DNA detected by *sctQ*-PCR. LAD: 10kb DNA ladder; NC: negative control; The gradient concentration started from 1 ng/µL to 1 fg/µL.

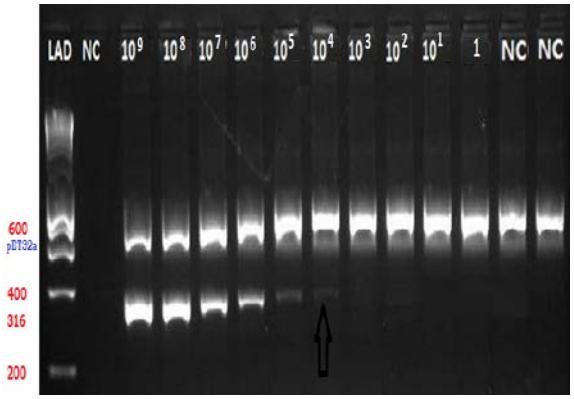

Figure 4. Gel electrophoresis of *duplex*-PCR performed on blood samples spiked with gradient inoculum of *B. pseudomallei*. The arrow indexes for the lower concentration of DNA detected by *sctQ*-PCR. LAD: 10kb DNA ladder; NC: negative control; The gradient inoculum started from 10^9 to 1 CFU/mL

4. Discussion

PCR-based diagnosis is preferred over immunoassays and shows higher sensitivity and specificity. A previously performed comparative study reported the superiority of PCR over three serological methods using samples obtained from culture-positive patients (Sermswan *et al*., 2000).

The complete sequence of the *B. pseudomallei* genome had been studied previously (Winstanley *et al*., 1999, Holden *et al*., 2004), and provided various gene targets for specific species identification using PCR assays including the type III secretion (TTS1) gene cluster (Winstanley and Hart, 2000), 16S–23S ribosomal RNA (rRNA) intergenic region (Kunakorn *et al*., 2000), rRNA spacer, 23S rRNA (Bauernfeind *et al*., 1998), 16S rRNA (Dharakul *et al*., 1996), lipopolysaccharide (LPS) gene (Rattanathongkom *et al*., 1997), flagellin C (*fliC*) and ribosomal protein subunit S21 (*rpsU*) (Tomaso *et al*., 2005).

Such genes have been utilized for the detection of *B. pseudomallei* from different clinical specimens and environmental samples, as well as pure culture, and showed good sensitivities and specificities (Brook *et al*., 1997, Rattanathongkom *et al*., 1997, Sura *et al*., 1997, Novak *et al*., 2006).

The selection of gene targets according to one strain may lead to false-negative results because a particular strain may not be representative for the global *B. pseudomallei* population (Tomaso *et al*., 2005).

On the other hand, PCR faces sensitivity and specificity issues when applied for a direct diagnosis on the patient's clinical specimens (Haase *et al*., 1998; Kunakorn *et al*., 2000; Sermswan *et al*., 2000).

Herein, the researchers reported the design, optimization, and validation of a PCR assay targeting the *sctQ* gene of the TTS1 cluster that is present among a wide range of *B. pseudomallei* strains.

For specificity testing, *B. thailandensis* and *B. cepacia* were included as relative non-*B. pseudomallei* species. *B. thailandensis* is well-known as the most genetically related to *B. pseudomallei*, and many of the pre-existing tests give false positive results (Zueter *et al*., 2016b), thus it would be of a high discriminative value for assay validation. *B. cepacia* is the most frequently isolated *Burkholderia* strain from human clinical samples along with *B. pseudomallei* (Pal 2018); therefore, it was added to the test panel to test the discriminative ability of *stcQ*-PCR.

The *sctQ*-PCR assay performance results agree with previous studies that reported excellent sensitivity and specificity of PCR when tested against purified bacterial DNA extracted from pure cultures or bacterial lysate. However, assay sensitivity decreased when testing clinical specimens due to possible effects of PCR inhibitors present in the specimens, applied specimen collection methods, or effects of antibiotics (Novak *et al*., 2006).

Many studies have evaluated different PCR assays for the detection of *B. pseudomallei* in clinical samples (Gal *et al*., 2005; Kaestli *et al*., 2012). Moreover, other comparative studies demonstrated the superiority of real-time PCR assays over conventional PCR in terms of greater analytical sensitivity, speed, and ease of use (Supaprom *et al*., 2007). Novak *et al* (2006) developed a real-time PCR assay targeting a type III secretion system for the identification of *B. pseudomallei*. They thoroughly evaluated the assay on spiked blood samples, and showed better accuracy than PCR assays previously published. Gal *et al*. (2005) have reported that the testing of a larger sample volume could improve the PCR performance for clinical samples.

A comparative study was performed for seven recently published real-time PCRs assays applied on clinical samples. The gene targets were *YLF/BTFC*, TTS1-*orf*2, TTS1-*orf*11, *IpxO*, and *mprA*, and showed that the real-time PCR assay targeting TTS1-*orf*2 was the most reliable on clinical samples with analytical and diagnostic specificity of 100 %, diagnostic sensitivity of 80 %, and a limit of detection of 5 fg/µL. In addition, the assay was useful in the detection of *B. pseudomallei* in purified samples and environmental samples (Kaestli *et al*., 2012).

5. Conclusion

PCR-based methods have significantly improved early disease diagnosis. Our developed assay showed acceptable performance compared to previous molecular assays. A larger panel of *Burkholderia species*, which was difficult to obtain in this study, is needed to confirm the discriminative ability of our assay. The end-point PCR designed in this study represents the first step on the way to the evaluation and commercialization of melioidosis molecular diagnosis in the hospital laboratory setting.

Acknowledgments

The researchers thank Dr. Khalid Hajissa and Nurleem Mursheed from the Microbiology Laboratory USM for their help procuring the isolates and for assistance with technical issues.

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JJBS

Jordan Journal of Biological Sciences

Volume 11, Number 4, September 2018
ISSN 1995-6673
Pages 441 - 449

Phytochemical Constituents and *in vitro* Antioxidant and Cytotoxic Activities of Different Extracts from the Aerial Parts of *Heliotrophium hirsutissimum* GRAUER

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Received February 8, 2018; Revised March 19, 2018; Accepted March 29, 2018

Abstract

The aim of this study is to detect the active phytochemicals in six different extracts namaly (diethyl ether, petroleum ether, ethyl acetate, methanol and, water (infusion and decoction) of the aerial parts of the *Heliotropium hirsutissimum* and to identify *in vitro* antioxidant and cytotoxic activities of these extracts. The phytochemicals present in the plant were assessed by standard methods. Six different extracts of *H. hirsutissimum* arial parts were tested for antioxidant activity using DPPH radical scavenging, H_2O_2 scavenging and metal chelating assays, cytotoxic activity using Brine Shrimp Lethality Assay. Phytochemicals found in the plant showed differences according to the extract type. As a result of the phytochemical screening of the aerial parts of the *H. hirsutissimum*, alkaloids, phenol, saponin, tannins and anthraquinones were detected. Flavonoid was not found in any of the extracts. The highest radical scavenging activity was found in the infusion extract, while the other extracts had low-free radical scavenging activity and H_2O_2 scavenging and metal chelating activities. It was determined that the different extracts obtained from *H. hirsutissimum* have no cytotoxic effects on *Artemia salina* nauplii compared with umbelliferone, which is a positive control at the applied concentration range (100 µg / mL-1000 µg / mL). The results demonstrated that the antioxidant properties of the *H. hirsutissimum* aerial-part extracts showed differentiation in the extract type and that none of the extracts have cytotoxic effects.

KeyWords: *Artemia salina*, Cytotoxic effect, DPPH scavenging activity, *Heliotropium hirsutissimum*, Phytochemical screening

1. Introductıon

The importance of medicinal plants and traditional health systems in solving health care problems around the world has gained much attention. Historically, all medicinal preparations were derived from plants, whether in the simple form of raw plant materials, or in the refined form of crude extracts, mixtures, etc. Recent estimates suggest that several thousands of plants have been known with medicinal applications in various crops (Farnsworth and Soejarto, 1991).

Natural products are the substances which originate from plants, animals, microbial and marine sources. Because of their vast availability in nature, they also play a significant role in the discovery of new therapeutic agents leading to the identification of bioactive molecules which allow the development of new pharmaceutical agents. İn addition they are used as a tool involved in the clarification of complex cellular and molecular mechanisms of actions of many biological and pathological processes (Ghori *et al*., 2016). Some plants have been subjected to the isolation of the active ingredients (chemical compounds) and their subsequent modification. These chemical compounds are able to perform physiological action in the body and are known as phytochemicals.─These have important health benefits, and can also be used to treat infections especially of microbial origin (Ranjaragan and Sathiyavani, 2014). A lot of plants and natural components have demonstrated antibacterial and wound-healing properties as well as anticancer activity, which confirms the potential for novel agents to be identified from uncharacterized natural plant resources (Holetz *et al*., 2002; Aridogan *et al*., 2002; Kaileh *et al*., 2007; Gonçalvez *et al.*, 2008; Martins *et al*., 2009) .

The family Boraginaceae is comprised of one-hundred genera and about two thousand species. The plants of this family are widely distributed in temperate, especially Mediterranean and tropical regions. *Heliotropium* is an important medicinal plant and is a large genus of the family Boraginaceae which consists of about 250-300 species around the whole World. Some of the taxa of this genus are *H. Bacciferum* Forssk., *H. europium* L., *H. baluchistanicum* K., *H. gillianum* R., *H. biannulatum* B., *H. Ovalifolium* Forssk., *H. Strigosum* Willd., *H. Eichwaldi* Steud., *H. indicum* L., *H.glutinosum* Phil., *H. sclerocarpum* Phil., *H. Sinuatum* Miers., *H. Subulatum* Hochst., *H. foertherianum* D. and *H. ovalifolium* Forssk, and *H, hirsutissimum Grauer* (Ghori et al., 2016). Plants of the genus *Heliotropium* display a wide range of pharmacological activities. Different biological activities of extracts and their bioactive constituents provide a basis

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for a better understanding of the underlying mechanisms involved (Singh *et al*., 2013). *Heliotropium* species have been used for their chloretic, antipyretic and cicatrizing activities (Baytop, 1999) and have been traditionally used for the treatment of gout, various inflammations, rheumatism, poisonous bites and skin diseases as a healing agent in various countries around the world. *Heliotropium* species are highly valued for antimicrobial and antioxidant activities due to the isolation of secondary metabolites like alkaloids, flavonoids and terpenoids. Because of these properties, *Heliotropium* can be used for the treatment of various bacterial and fungal infections in modern medicine as confirmed by folk medicinal studies. In addition, the anti-inflammatory, antiviral, antitumor, antidiabetic and antihyperlipidemic as well as the gastroprotective activities also enhance the medicinal value of *Heliotropium* in the future (Ghori *et al.*, 2016). Due to the broad range importance of the ethno-pharmacological flora, this study was arranged to collect ethno-medicinal knowledge about *H. hirsutissimum.*

They are known to contain pyrrolizidin alkaloids (heliotrine, lasiocarpine, europine, supinine) (Güner, 1986). The Pyrrolizidine alkaloids that are abundantly found in *Heliotropium* are responsible for its poisonous nature such as hepatotoxicity, mutagenicity and hepatocarcinogenicity. The toxic nature of pyrrolizidine alkaloids can be attributed to different reasons. The plants which are the main source of these alkaloids are consumed in food, and are sometimes used in the form of herbal medicines (Ghori *et al.*, 2016). However, no toxicological or pharmacological studies have been carried out in detail so far on this plant, with the exception of a recent study which explored its antioxidant properties.

Brine Shrimp (*Artemia salina* L.) bioassay is considered as a preliminary screening for the presence of antitumor compounds and is used to determine the plant extract toxicity (Meyer *et al*., 1982). Using Brine Shrimp larvae, pharmacognosists and natural-product chemists were able to detect and isolate the plant constituents and its active compounds with a variety of pharmaceutical activities (Alali *et al*., 2008). *Brine shrimp* is considered a rapid, inexpensive, and in-house bioassay for screening and fractionation monitoring of physiologically active plant extracts (Jayasuriya *et al*., 1989). *Brine shrimp* is utilized previously in various bioassay systems (Meyer *et al*., 1982; Ratnayake *et al*., 1992). According to Meyer *et al*. (1982) several extracts derived from naturall products which had LC50 $\leq$ 1000 μg/ml using *Brine shrimp* bioassay were known to contain physiological active principles (Meyer *et al*., 1982).

The present study is aimed at evaluating the phytochemical constituents, antioxidant potential and free radical-scavenging capacity of different extracts of the *Heliotropium hirsutissimum* Grauer (Boraginaceae) and the cytotoxic activities of these extracts on developing brine shrimp nauplii. The activities have been selected because of their great medicinal relevance. Over the last years, interest in the antioxidant activity of plant extracts has become larger and very important due to the fact that free radicals (e.g. reactive oxygen species (ROS) can be responsible for various diseases including heart diseases, strokes, arteriosclerosis and cancer, as well as the aging process.

2. Materials and Methods

2.1. Plant Collection and Identification

The fresh aerial parts of *Heliotropium hirsutissimum* were collected from the surrounding areas of Adnan Menderes University Central Campus, Aydın, Turkey, during Agust of 2012. The plant was recognized by its local name and was then clarified by Dr. Özkan Eren of Adnan Menderes University, Art and Science Faculty, Department of Biology, Aydın, Turkey. A voucher specimen of the plant has been deposited (AYDN-2266) in the herbarium for further reference. Fresh healthy flowers, leaves and stems, thoroughly washed (2-3 times) with water and were dried in the shade room at the ambient temperature for two to three weeks. The dried plants were finely ground with a mixer and were stored in the dark at room temperature in closed containers until further use.

2.2. Extraction of the Plant Material

Dried ground whole plants of *H. hirsutissimum* were extracted with a solvent series of increasing polarity (diethyl ether, petroleum ether, ethyl acetate, methanol, and water (infusion and decoction) extracts). For extractions, 60 g of plant materials were used. 600 mL of solvent was added to 60 g of the plant material. After completing the first soxhlet extraction with diethyl ether (at 40°C for approximately twelve hours, until the solvent became colorless) and filtration, the plant material was dried and subjected to the second extraction with petroleum ether, the third extraction with ethyl acetate, and the fourth extraction with methanol (Goffin *et al*., 2003; Lee *et al*., 2003; Miliauskas *et al*., 2004; Avcı *et al*., 2006). The extracts were evaporated and yielded 1.162, 0.954, 1.678 and 4.920 g dried mass, respectively. After the methanol extraction, the plant material was dried and subjected to the water (infusion) extraction. For water (infusion) extraction, 600 ml distilled water at 80°C was added to the plant material for ten minutes and the extract was filtered. For the second water extraction (decoction), 600 ml of distilled water was added to 60 g of dried plant material and boiled for ten minutes, and extract was filtered (Ljubuncic *et al*., 2005). The filtered extracts were lyophilized and yielded 1.811 and 2.507 g dried mass, respectively. The extracts were sealed in glass bottles and stored at -20°C until use.

2.3. Phytochemical Screening for Six Different Extracts

Phytochemical tests of the six different extracts were carried out to detect the presence of particular compounds using a standard procedure. These concentrations were selected according to the data obtained from the preliminary experiments. The extracts were subjected to preliminary phytochemical testing for the detection of major chemical groups (Table 1). The details of the tests as are follows:

2.3.1. Detection of Phenols

Ethyl acetate and methanol extracts prepared in ethanol were spotted on a filter paper. A drop of phosphomolybdic acid reagent was added to the spots and was exposed to ammonia vapors. Blue coloration of the spots indicates the presence of phenols (Ravishankara *et al*., 2002).

2.3.2. Detection of Tannins

To 2-3 mL of methanolic extract, 10 % alcoholic ferric chloride solution was added. Dark blue or greenish grey coloration of the solution indicates the presence of tannins in the extracts (Ravishankara *et al*., 2002).

2.3.3. Detection of Alkaloids

A drop of extracts prepared in methanol was spotted in a small piece of precoated TLC plate, and the plate was sprayed with Dragendorff's reagent. Orange coloration of the spot indicates the presence of alkaloids (Ravishankara *et al*., 2002).

2.3.4. Detection of Anthraquinones

About 50 mg of the extracts was heated with 10 % ferric chloride solution and 1 mL of concentrated hydrochloric acid. The extracts were cooled, filtered, and the filtrates were shaken with diethyl ether. The ether extracts were further extracted with strong ammonia. Pink or deep red coloration of aqueous layer indicates the presence of anthraquinones (Ravishankara *et al*., 2002).

2.3.5. Detection of Saponins

10 mg of the extracts were mixed with hot water, and the mixtures were shaken for thirty seconds. The formation of a stable foam indicates the presence of saponins (Dominguez, 1973).

2.3.6. Detection of Flavonoids

To 2-3 mL of the extracts prepared in methanol, a piece of magnesium ribbon and 1 mL of concentrated hydrochloric acid were added. Pink-red or red coloration of the solution indicates the presence of flavonoids (Ravishankara *et al*., 2002).

2.4. Determination of Total Phenolic Content

The total phenolic compound contents in the extracts were determined by the Folin-Ciocalteu method (Singleton *et al*., 1999). 100 µL of the properly diluted extract solutions were mixed with 1 mL of FC reagent. The reagent was pre-diluted, ten times, with distilled water. After standing for three minutes at room temperature, 3 mL of (2 % w/v) a sodium carbonate solution was added. The solutions were mixed and allowed to stand for two hours at the room temperature. Then, the absorbance was measured at 760 nm, using a UV–visible spectrophotometer (Shimadzu PharmaSpec UV-1700, Japan). A calibration curve was prepared using a standard solution of gallic acid (20, 40, 60, 80 and 100 mg/L). Results were expressed as mg gallic acid equivalents/g of sample.

2.5. Determination of Antioxidant Activity

2.5.1. DPPH Free Radical Scavenging Assay

The assay for DPPH free radical scavenging potential is based on the scavenging activity of stable 1,1-diphenyl-2-picryl-hydrazyl radical (DPPH). The free radical scavenging activity of diethyl ether, petroleum ether, ethyl acetate, methanol and water (infusion and decoction extracts) extracts from *H. hirsutissimum* was tested for their ability to bleach the stable 1,1-diphenyl-2-picryl-hydrazyl radical (DPPH) (Brand-Williams *et al*., 1995). One milliliter of 0.1 mM DPPH methanol solution was added to 3 mL of various concentrations of the extracts in methanol. The mixture was shaken vigorously and was left at room temperature. After thirty minutes, the absorbance of mixture was measured at λ=517 nm. Tests were carried out in triplicate. Rutin (100 ppm), a citrus flavonoid glycoside, was used as a standard, and the Shimadzu PharmaSpec UV–1700 UV-Visible spectrophotometer was used for measurement. Finally, the DPPH radical scavenging activity of the extracts was calculated using the following equation:

Scavenging capacity % = 100 - [(Ab of sample-Ab of blank) × 100/Ab of control]

where Ab control is the absorbance of $DPPH^{\bullet}$ solution without extracts.

The extract concentration providing 50 % inhibition (IC_{50}) was calculated from the plot of inhibition percentage against extract concentration.

2.5.2. H_2O_2 Scavenging Assay

Hydrogen peroxide scavenging activities of the extracts from *H. hirsutissimum* were determined by the method described by Ruch *et al.* (1989). A solution of H_2O_2 (40mM) was prepared in phosphate buffer (pH 7.4). The reaction mixtures contained 40 mM of H_2O_2 and different concentrations of the extracts, and the absorbance values were measured after ten minutes using wavelength of 230 nm. Ascorbic acid was used as the standard.

2.5.3. Metal Ion Chelating Ability

The chelation of ferrous ions by extracts was estimated by method of Dinis *et al.,* (1994). Briefly, 50 µL of 2 mM $FeCl_2$ was added to 1 mL of different concentrations of the extracts. The reaction was initiated by the addition of 0.2 ml of 5 mM ferrozine solution. The mixture was vigorously shaken and left to stand at room temperature for ten minutes. The absorbance of the solution was thereafter measured at 562 nm. The percentage inhibition of ferrozine-Fe^{2+} complex formation was calculated according to the following formula:

$$[(A_0 - As)/ As] \times 100$$

where A_0 is the absorbance of the control, and As is the absorbance of the extract/ standard. Na_2EDTA is used as positive control (standard).

2.6. Cytotoxic Activity

The cytotoxicity of the *H. hirsutissimum* extracts was evaluated by *Artemia salina* lethality test according to the procedure described by Solis *et al.* (1993). Brine shrimp eggs were hatched in artificial sea water prepared from commercial sea salt 40 g/L. The compartments plastic chamber are used, the eggs were sprinkled into the compartment which was darkening. After forty-eight hours of incubation at room temperature (25-29°C), nauplii were collected by pipette from the lighted side of the chamber, and were exposed to a 60-W lamp, pH 8.8.

The extracts were dissolved in DMSO (up to 2 % of final dosage), and were diluted with sea water. Serial dilutions were made in the wells of 96-well microplates in triplicate in 500 µL sea water. Control wells with DMSO were included in each experiment. A suspension of nauplii containing 10-15 organisms (100 µL) was added to each well. The plates were covered and incubated at room temperature (25-29°C) for five and twety-four hours.

From 20 mg of each extract, 1, 10, 100 and 1000 ppm solutions were prepared in triplicate. Then, ten specimens

with forty-eight hours of hatching in sea water and distilled water (1:1) were placed in each tube and three negative control tubes (saline solution and DMSO 1 %). Appropriate volumes of the saline solution in tubes were added until 5 mL of the saline solution containing 10 nauplii each to obtain the final sample concentrations. After twenty-four hours, the plates were then examined under the binocular stereomicroscope, and the numbers of dead (non-motile) nauplii in each well were counted. The percentage of lethality of the nauplii for each concentration and control was calculated. Umbelliferon was used as positive control and salt water was used as negative control. For each plate, the number of the dead and of live nauplii, was counted and % death was determined:

$$\%Death = \frac{\text{Number of dead nauplii}}{\text{Number of dead nauplii + Number of live nauplii}} \times 100$$

2.7. Statistical Analysis

The number of deaths was counted and the results were tabulated and submitted to probit analysis in SPSS® 11.5 software (IBM® Corp., NY, USA), obtaining the value of LC_{50} with a 95 % confidence interval. Each experiment was carried out triplicate. Statistically significant difference was considered at the level of $p<0.05$.

3. Results

3.1. Phytochemical screening

Results of phytochemical screening are presented in Table 1. Phenols were detected in the petroleum ether and water extracts (infusion and decoction) but not in diethyl ether extract, ethyl acetate and methanol extract of *H. hirsutissimum*. Tannins were present only in the methanol extract. A very small amount of Tamins was determined in the diethyl ether extract but not in the other extracts. Alkaloids were detected in the diethyl ether, petroleum ether, ethyl acetate and decoction extracts, but not in the methanol extract. Alkaloids were determined with a very small amount in the infusion extract. Anthraquinones were detected in the ethyl acetate and methanol extracts. Saponins were present in the ethyl acetate and methanol extracts. Flavonoids were not detected in any of the extracts.

Table 1. Qualitative analysis of the phytochemicals of different extracts of *H. hirsutissimum*

Phytochemicals	Extracts					
	Diethyl ether extract	Petroleum ether extract	Ethyl acetate extract	Methanol extract	Aqueous extract (Infusion)	Aqueous Extract (Decoction)
Phenols	-	+	-	-	+	+
Tannins	*	-	-	+	-	-
Alkaloids	+	+	+	-	*	+
Anthraquinones	-	-	+	+	-	-
Saponins	-	-	-	+	+	+
Flavonoids	-	-	-	-	-	-

+ present; *: slightly present; -: absent

3.2. Determination of Total Phenolic Content

The total phenolic content in the examined plant extracts is expressed in terms of gallic acid equivalent (the standard curve equation: $y = 0.003x + 0.001$, $r2 = 0.9975$). The total phenolic content values are expressed as mg of GA/g of extract (Table 2).

Table 2. Total phenolic contents of *H. hirsutissimum* extracts expressed in terms of gallic acid equivalent (mg of GA/g of extract)

Extracts	mg of GA/g of extract
Diethyl ether	27.6
Petroleum ether	27.7
Ethyl acetate	47.7
Methanol	31.0
Infusion	23.3
Decoction	30.0

Each value is the mean of three analyses

3.3. Antioxidant Activity

3.3.1. DPPH Scavenging Activity

The antioxidant activities of six different extracts are expressed in terms of the percentage of inhibition (%) and IC_{50} values (μg/mL) (Table 3). Parallel to the examination of the antioxidant activity of the plant extracts, the values for standard compounds were obtained and compared to the values of the antioxidant activity. The standard substance was rutin. A lower value of EC_{50} indicates a higher antioxidant activity. Antioxidant activities of the extracts showed different values. Extracts showed a concentration-dependent radical scavenging activity. The highest activity to neutralize DPPH radicals was found for infusion extract, which neutralized 50 % of free radicals at the increased concentration (30.5 ± 0.019 for 25 ppm; 52.8 ± 0.00 for 50 ppm and 74.4±0.16 for 100 ppm, repectively). A moderate activity was found for methanol, and decoction extracts. Due to the low activity of diethyl ether, petroleum ether, ethyl acetate, and decoction extracts, IC_{50} are not calculated for these extracts.

Table 3. Antioxidant (DPPH scavenging) activity of six different extracts of *H. hirsutissimum*

Extracts	Concentrations (ppm)	DPPH scavenging activity (%±SD)
Diethyl ether	25	0.00±0.0025
	50	0.00±0.0025
	100	15.8±0.0025
Petroleum ether	25	0.00 ±13.27
	50	0.00 ±0.005
	100	11.0±0.030
Ethyl acetate	25	0.00 ±0.0076
	50	0.00 ±0.0023
	100	12.1±0.025
Methanol	25	21.2±0.021
	50	30.5±0.0005
	100	43.6±0.000
Infusion	25	30.5±0.0190
	50	52.8±0.0005*
	100	74.4±0.1646*
Decoction	25	10.7±0.0076
	50	14.6±0.010
	100	19.6±0.0020
Rutin	100 ppm	89.1±0.0015*

*****p <0.05

3.4. H_2O_2 Radical Scavenging Activity

H_2O_2 scavenging activities (%) of different concentrations of different extracts are shown in Table 4. H_2O_2 scavenging activity of diethyl ether extract was observed at 5 and 10 ppm concentrations (33.1 % and 14.19 %, respectively), but was not observed at 20 ppm concentration. Petroleum ether and methanol extracts showed moderate H_2O_2 scavenging activity. Other extracts showed low H_2O_2 scavenging activity at tested concentrations (5, 10 and 20 ppm).

Table 4. H_2O_2 scavenging and metal chelating activity of six different extracts of *H. hirsutissimum*

Extracts	Concentrations (ppm)	H_2O_2 scavenging activity (%±SD)	Metal chelating activity (%±SD)
Diethyl ether	5	33.1±0.0016	0.00±0.0215
	10	14.19±0.0023	0.00±0.0015
	20	0.00±0.0008	0.00±0.0090
Petroleum ether	5	44.9±0.0043	0.00±0.0120
	10	41.5±0.0012	0.00±0.0045
	20	23.7±0.0043	23.4±0.0020
Ethyl acetate	5	28.3±0.0043	0.00±0.0010
	10	14.0 ±0.0019	0.00±0.0000
	20	0.00±0.085	0.00±0.0010
Methanol	5	41.3 ±0.0071	23.4±0.0020
	10	20.9±0.0005	0.00±0.0055
	20	14.3±0.0009	10.1±0.0005
Infusion	5	31.7±0.0008	0.00±0.0090
	10	16.1±0.0012	0.00±0.0000
	20	27.8±0.0019	36.1±0.0010
Decoction	5	31.2±0.0022	0.00±0.0050
	10	22.2±0.014	0.00±0.0045
	20	15.3±0.0015	0.00±0.0025
Ascorbic acid	2µg/ml	71.8±0.0069*	
EDTA	2µg/ml		100.0±0.0000*

*****p<0.05

3.5. Metal Ion Chelating Activity

The metal ion chelating activity of six different extracts of *H. hirsutissimum* extracts was determined at 5, 10 and 20 ppm concentrations, and the results are depicted in Table 4. Although standard agent EDTA, showed a high ion metal chelating potential (100 %), the extracts showed a low chelating activity. The metal chelating activity was 36.1 % (20 ppm) in the infusion extract, 23.4 % (5 ppm) in the methanol extract and 23.4 % (20 ppm) in the petroleum ether extract. Other extracts did not show metal chelating activity.

3.6. Brine Shrimp Lethality Assay

The results of lethal effects of the extracts to brine shrimp larvae (% mortality at different concentrations and LC_{50} values) were shown in Table 5. An approximate linear correlation was observed when logarithm of concentration versus percentage of mortality was plotted on the graph paper, and the values of LC_{50} were calculated using Microsoft Excel 2003**.** According to Nguta *et al.* (2011), crude extracts with LC_{50} values less than 100 ppm are considered highly toxic. Those with LC_{50} values between 100 ppm and 500 ppm are moderately toxic and the ones with LC_{50} values between 500 ppm and 1000 ppm, are considered mildly toxic. Those with LC_{50} values above 1000 ppm are considered non-toxic.

As a result of evaluating the extracts' data by Probit analysis; it was found that the tested extracts did not reach the LC_{50} values at the determined concentration ranges (10, 25, 50, 100, 250, 500 and 1000 ppm). Of the six tested extracts, those with LC_{50} between 10 and 1000 ppm showed weak toxicity. LC_{10} value results are: (p<0.05) showed no significant difference in the other extracts except methanol (LC_{10} = 258.610 ppm) and diethyl ether (LC_{10} = 93.344 ppm). The extracts showed weak cytotoxic activity with LC_{50} value compared with the activity of standard with LC_{50} value (Table 5).

Table 5. Effects of the six different extracts of *H. hirsutissimum* on the *Brine Shrimp* nauplii (*Aretmia salina)* after a 24-hr exposure

Extracts	Time (h)	LC_{10} (ppm)	LC_{50} (ppm)
Diethyl ether	24	93.344	>1000
Petroleum ether	24	>1000	>1000
Ethyl acetate	24	>1000	>1000
Methanol	24	258.610	>1000
Infusion (water)	24	>1000	>1000
Decoction (water)	24	>1000	>1000
Umbelliferon (positive control)	24	53.773	170.836

4. Discussion

Herbal products are considered an important source of potentially useful compounds for the development of new phytotherapeutic agents. *Heliotropium* has been traditionally used for the treatment of gout, various inflammations, rheumatism, poisonous bites, and skin diseases as a healing agent in various countries around the

World (Ghori, 2016). The *Heliotropium* species grow in arid regions with extreme environmental conditions and produce a resinous exudate from the trichomes that cover its foliar surface and stems. Phytochemical research revealed that this exudate is constituted by a mixture of different compounds, mainly flavonoids and aromatic geranyl derivatives **(**Villarroel, 1991; Tores et al., 1994; Urzua et al., 2000; Modak, 2003). A variety of constituents are identified and isolated from different species of the genus *Heliotropium* which are phytochemically active and have significant therapeutic effects. Many classes of organic compounds such as pyrrolizidine alkaloids (PAs), phenolic compounds, terpenoids, and quinones are very abundantly present in *Heliotropium. Heliotropium* species are highly valued for their antimicrobial and antioxidant activities due to the isolation of secondary metabolites such as alkaloids, flavonoids and terpenoids (Ghori et al., 2016).

The phytochemical screening in the present study, has revealed the presence of phenols, tannins, alkaloids, flavonoids, anthraquinones, and saponins in six different extracts of *H. hirsutissimum* (Table 1). Phenols were detected in the petroleum ether and water extracts (infusion and decoction) but not in diethyl ether, ethyl acetate and methanol extracts of *H. hirsutissimum*. Tannins were present in the methanol extract and were determined as of a very small amount in the diethyl ether extracts, but not in the other extracts. Alkaloids were detected in the diethyl ether, petroleum ether, ethyl asetate, and the water extracts (decoction) and with very small amounts in the infusion extract, but not in the methanol extract. The inability to observe alkaloids in the methanol extract may be due to the fact that the alkaloids are not soluble in methanol or may be exposed to the antagonistic effect of any substance contained therein. Alkaloids play some metabolic role and control development in the living system. They are also involved in protective functions in animals, and are used as medicine especially the steroidal alkaloids (Lalitha *et al*., 2012). Phenolics and alkaloids detected in the extracts are compounds that have been documented to possess medicinal properties (Salah *et al*., 1995; Obdulio *et al*., 1997; Okwu, 2004; Liu, 2004; Harini *et al*., 2014). The flavonoids and phenolic compounds in plants have been reported to exert multiple biological effects including antioxidant, free radical scavenging abilities, anti-inflammatory, anti-carcinogenic etc. (Lalitha *et al*., 2012). The presence of alkaloids has also been identified in other *Heliotropium* species (Reina *et al*., 1998; Souza et al., 2005; Osungunna and Adedeji, 2011). Previous studies have reported that pyrrolizidine alkaloids, which are a class of alkaloids, are present in almost all species belonging to the Boraginaceae family. Anthraquinones were detected in the ethyl acetate and methanol extracts. Saponins are present in the ethyl acetate and methanol extracts (Table 1). In the current study, saponins were found to be the most common content observed after alkaloids in the plant extracts. Previous studies have also found saponins in the methanol extract of the *H. indicum* (Sharma and Alexander, 2011). The results of this study are also in accordance with the literature.

The total phenolic contents in the *H. hirsutissimum* extracts depend on the type of the extract, i.e. the polarity of the solvent used in the extraction. The high solubility of phenols in polar solvents provides high concentration of these compounds in the extracts obtained using polar solvents for the extraction (Zhou and Yu, 2004; Mohsen and Ammar, 2008). The variation may be due to environmental conditions, which can modify the constituents of the plant.

Comparatively, the stable organic radical DPPH has been broadly utilized in the determination of the antioxidant activity of different plant extracts as well as purified compounds (Yen and Duh, 1994; Brand-Williams, *et al.*, 1995). The ability of antioxidants for DPPH radical scavenging is supposed to be due to their hydrogen donating property (Soares *et al.*, 1997). After acceptance of an electron or a hydrogen atom, a stable diamagnetic molecule will emerge which results in the vanishing of the absorption band at 517 nm. The radical scavenging activity of the samples corresponds to the remaining DPPH in an inverse manner. Numerous antioxidant methods have been proposed to evaluate the antioxidant activity. Of these, the total antioxidant activity, reducing power, DPPH assay, metal chelating, active oxygen species such as H_2O_2, $O^{\cdot-}$ and $OH^{\cdot}$ quencing assays are the most commonly used in the evalution of antioxidant activities of plant extracts.

Different extracts of *H. hirsutissimum* showed low DPPH scavenging activity whereas other *Helioptrium* species showed strong antioxidant activity. The plant, *H. strigosum*, showed excellent DPPH scavenging activity. The antioxidant activity was shown by other plants of the genus, *Heliotropium* (Begüm, 2014). *H. taltalense* and flavonoids islolated exhibited antioxidant activity which suggest that *H. strigosum* may possess flavonoids responsible for the antioxidant activity (Modak *et al*., 2009). Similarly, the ethyl acetate fraction also showed DPPH scavenging activity and is consistent with the antioxidant activity shown by *H.sinuatum.* Modak *et al*., (2003) islolated, 4-(3',5'-dihydroxynona-decyl) phenol 1, and eight flavonoids from *H. sinuatum* (Modak *et al*., 2003), and *H. strigosum* (Hussain *et al*, 2010) reported the antioxidant activity of these compounds. The aqueous and organic extracts from the same plant showed different activities, the organic extracts showed the same or greater activity than the aqueous extracts, these results suggest that the interesting active compounds in this plant have a limited solubility in water, and are expected to be non-polar hydrophobic organic compounds (Jadarat *et al.*, 2014).

Hydrogen peroxide is a weak oxidizing agent, and can inactivate a few enzymes directly, usually by the oxidation of essential thiol (-SH) groups. Hydrogen peroxide itself is not very reactive, but sometimes it can be toxic to cells because of the increase in the hydroxyl radicals in the cells. It can cross cell membrane rapidly, once inside the cell, H_2O_2 can probably react with Fe^{2+} and possibly Cu^{2+} ions to form hydroxyl radical, and this may be the origin of many of its toxic effects (Halliwell, 1991; Mohan *et al*., 2012). When the results of this study were compared with ascorbic acid, six different extracts of *H. hirsutissimum* did not show H_2O_2 scavenging activity except the petroleum ether extract. (Table 4). Another *Helioptrium* species, *H. ramosissium*, has a strong H_2O_2 scavenging ability (Shatat *et al*., 2015). Also, the extract of *H. indicum* leaves exhibits the greatest antioxidant activity through the scavenging of free radicals.

The iron-chelating capacity test measures the ability of antioxidants to compete with ferrozine in chelating ferrous ion (Elmastas *et al*., 2006). Ferrozine can quantitatively form complexes with Fe^{2+}. In the presence of samples possessing chelating activity, the formation of complexes is decreased. Therefore, measurement of the rate of color reduction helps to estimate the chelating activity of the samples. *H. indicum* leaves exhibited the greatest iron chelator and iron reducing power. Phytochemicals of *H. indicum* leaves exhibited the greatest antioxidant activity DPPH, superoxide anion scavenging and metal chelator (iron chelator and iron reducing power). When the results of this study were compared with other *Heliotropium* species, the antioxidant activity, phenolic content, H_2O_2 scavenging and metal cheathing activity of the *H. hirsutissimum* extracts were different. This may be due mainly to the extract of *H. hirsutissimum* being a crude extract with active compounds that are not purified. However, the active compounds of the *H. Hirsutissimum* extracts will be purified and identified in the future in an ongoing research in the laboratory.

The brine shrimp lethality assay is a general bioassay that seems to be capable of detecting a wide spectrum of bioactivity present in the crude extracts. The commercial availability of inexpensive brine shrimp eggs, the low cost, the safety and ease of performing the assay, as well as the requirement of no special technology all make this a very helpful bench-top tool for the phytochemistry laboratory. The lethality to brine shrimp is recommended as an effective prescreen to the existing cytotoxicity and antitumor assays. A number of studies have established the use of the brine shrimp assay to screen plants commonly used as pesticides, anticancer, and with molluscicidal, larvicidal, fungicidal, and cytotoxic activity (Khalighi-Sigaroodi *et al*., 2012). In order to study the toxicity of these medicinal ponctuation, we performed brine shrimp lethality bioassay based on the ability to kill *Artemia naupli* in laboratory condition. Substances submitted to this bioassay, which lead to the death of half of the specimens at a lethal concentration of up to 1000 ppm, are considered active. They were subsequently tested in these trials, obtaining a good correlation (McLaughlin and Rogers, 1988; Arcanjo *et al*., 2012).

The results showed no cytotoxic properties of the six different extracts obtained from *H. hirsutissimum* on *Artemia salina* larvae (Table 5). The lethality of the crude extracts (LC_{50} value less than 100 ppm) to brine shrimps indicates the presence of potent cytotoxic compounds which necessitate further investigation (Alam, et al., 2011). Shah *et al.,* (2015) have shown that *H. strigosum* and its organic fractions exhibit cytotoxic and phytotoxic activities against *Artemia salina* Leach. The ethyl acetate and chloroform fractions showed marked cytotoxicity action. Ethyl acetate and chloroform fractions were more potent for the evaulated toxicity effects. The ethyl acetate and chloroform fractions showed marked cytotoxicity action. In the phytotoxicity study, ethyl acetate was the most potent, followed by chloroform. Researchers have suggested that studies should be done to isolate active compounds in the cytotoxic properties of the extract (Shah *et al*., 2015).

5. Conclusion

On the basis of the results obtained this study confirms the cytotoxic activity of different exracts of *H. hirsutissimum* against *Brine shrimp* larvae. When the results of this study were compared with other Heliotropium species, the antioxidant activity, phenolic content, H_2O_2 scavenging and metal cheathing activity of the *H. hirsutissimum* extracts were found different. These differences may be due to the parts of the plant used, climate and soil differences. Furthermore, this study found that the plant extracts have a broad spectrum of activities. Nonetheless, activity-directed assay is necessary on this plant with a view to isolating and characterizing the active metabolite responsible for the observed activity. Finally, this study can be used as a basis for utilizing this plant species for further investigation in drug discovery for potential new natural bioactive compounds.

Acknowledgements

The researchers are thankful for Adnan Menderes University Scientific Research Foundation for their financial support (Project No: FEF-13017). Thanks are also extended to Dr. Özkan EREN for the botanical identification of *Heliotropium hirsutissimum* and for Dr. Özlem Sultan ASLANTÜRK for her helpful advice during writing this manuscript

Conflıct of Interests

Authors have declared that no conflict of interests exists

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Jordan Journal of Biological Sciences

Volume 11, Number 4, September 2018
ISSN 1995-6673
Pages 451 - 459

Bacillus atrophaeus A7 Crude Chitinase: Characterization and Potential Role against *Drosophila melanogaster* Larvae

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Received March 1, 2018; Revised April 13, 2018; Accepted April 21, 2018

Abstract

Microbial chitinases are important environmental biomolecules with biotechnological and medicinal applications in addition to being a source of environmental friendly biopesticides. They are considered as safe alternatives to some available chemical insecticides, especially against insects that may act as an intermediate hosts as well as vectors between manifested plant materials and humans. A crude chitinolytic enzyme was isolated from isolate A7 (*Bacillus atrophaeus* Nakamura 1989). The isolate was identified based on the morphological and biochemical characteristics as well as the sequencing of 16S rRNA. The produced enzyme had a total activity of 68.9±1.03 mU/mL; a specific activity of 2670±40.2 mU/mg protein, and was optimally active at 40°C, 4-9 pH with stability for one hour at 30-40°C and 6-7 pH. It was inhibited by Cu^{2+}, Fe^{3+}, Ni^{3+}, Zn^{2+} and Ba^{2+} metal ions and impeded the development of 50 % of *Drosophila melanogaster* larvae into adults (LD_{50}) at 17.3±1.4 mU/mL. In this study, the larvicidal activity of chitinase from *B. atrophaeus* is explored for the first time with the potential of being applied as environmental friendly biopesticide technologies.

Keywords: *Bacillus atrophaeus*, *Drosophila melanogaster*, Chitinase enzyme, Chitinolytic bacteria, Larvicidal activity, Bioinsecticides

1. Introduction

Chitin is a naturally abundant biomolecule after cellulose. It accumulates as a waste from shellfish production and processing industries in the terrestrial environment (Chakrabortty *et al*., 2012). It occurs mainly as a structural component in the exoskeleton of arthropods and to lesser extents in plants, fungi, bacteria and other animals (Zarei *et al*., 2012). It could be degraded into various chitooligomer molecules that may undergo further enzymatic breakdown generating N-acetylglucosamine (GlcNAc) monomers by sequential action of two types of chitinase enzymes: endochitinase and exochitinase. Endochitinase (EC 3.2.1.14) randomly catalyzes the cleavage of β-1,4-glycosidic bonds in chitin to release N-acetylchitooligosaccharides. On the other hand, exo-chitinase (EC 3.2.1.52, includes the formerly classified EC 3.2.1.29 and EC 3.2.1.30) catalyzes the progressive release of diacetylchitobiose starting at the non-reducing end of the chitin that is further cleaved to generate monomers of GlcNAc (Fu *et al*., 2014). Chitinases are receiving an increased attention due to their broad range of applications. They are potential biocontrol agents against plant-pathogenic fungi (Nguyen *et al*., 2015) and control insect pests (Rathore and Gupta, 2015). They have a role in the bioconversion of chitin to single cell protein from shellfish waste (Hao *et al*., 2012) and in the isolation of protoplast from fungi (Dahiya *et al*., 2005), in addition to their medical applications (Aam *et al*., 2010; Stoykov *et al*., 2015).

Drosophila melanogaster is considered an insect pest that infests various habitats where fermenting fruits are found. In fact, infestation removal is difficult as the larvae may continue to develop in fermenting fruit even if the adult population was eliminated. Moreover, *D. melanogaster* could act as an intermediate host as well as a vector between manifested plant materials (Keesey *et al*., 2017) and a source of foodborne disease outbreaks among humans (Blazar *et al*., 2011). Hence, looking for a biopesticide as an environmental friendly alternative is of primary concern, such as proteolytic and chitinase enzymes.

Chitinolytic microbes occur widely in nature. They are preferred sources of chitinase because of their low production cost and the availability of raw materials for their cultivation (Singh *et al*., 2008). Their chitinases have nutritional and morphogenetic roles in fungi (Adams, 2004), and play a role in the utilization of chitin as a carbon and energy source and in the recycling of nitrogen in nature (Chandran *et al*., 2007). Some of the best-known Gram-negative and Gram-positive bacterial genera are remarkably producers of chitinolytic enzymes including *Serratia*, *Aeromonas*, *Pseudomonas*, *Alteromonas*, *Enterobacter*, *Bacillus*, *Clostridium*, *Micronomospora*, and *Actinomyces* (Cheba *et al*., 2016). Intriguingly, some of

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them were reported to produce multiple forms of chitinases with different molecular masses (Anuradha and Revathi, 2013).

Herein, *Bacillus atrophaeus* A7 was isolated from exoskeleton of dead ground beetles (Coleoptera: Carabidae) and was selected as chitinolytic bacterium. *B. atrophaeus* is noted as a bio-agent against various fungal pathogens, in addition to having the capacity to improve crop production acting as plant growth stimulants as well as the role in other biotechnological approaches (Liu *et al*., 2012; Zhang *et al*., 2013; Sella *et al*., 2015). Recently, it was reported that *B. atrophaeus* HAB-5 exhibited a broad antifungal spectrum, particularly against *Colletotrichum* sp., *Alternaria* sp. and *Fusarium* sp. with activity under a wide pH range (pH 2-12), and heat stability between 40 °C and 100 °C and pH 5 to 10 (Rajaofera *et al*., 2017).

Nevertheless, the insecticidal activity of chitinases from *B. atrophaeus* has not been previously reported. Therefore, the produced chitinase from *B. atrophaeus* A7 was evaluated in term of its activity, stability under various conditions as well as its potential role as an insecticidal agent against *D. melanogaster.*

2. Materials and Methods

2.1. Screening and Isolation of Producing Bacteria.

Bacillus atrophaeus A7 was isolated, among other bacterial isolates, from the exoskeletons of dead beetles *Dixus* sp. (Coleoptera: Carabidae) collected from different locations in AL-Tafilah Province/South of Jordan. The beetle was identified based on the annotated checklist by Nasir and Katbeh-Bader (2017). It was in a dried out condition with decayed internal body mass (thorax and abdominal regions) and intact exoskeleton. The exoskeletons were pulverized using sterilized mortar and pestle, and were spread on top of chitin agar plates of the following composition (g/L): 0.6 % Na_2HPO_4, 0.3 % KH_2PO_4, 0.1 % NH_4Cl, 0.05 % NaCl, 0.005 % yeast extract, 3 % colloidal chitin and 1.8 % agar, pH 7.2. The agar plates were incubated for five days at 27ºC with continuous observation. Bacterial colonies forming clear zones due to chitin hydrolysis were selected and purified on new chitin agar plates. The purified bacterial isolates were transferred to 30 % (v/v) glycerol solution and were stored at -20ºC until the date of use.

Colloidal chitin was prepared as described in Hsu and Lockwood (1975) with minor modifications. Twenty grams of grounded crab shell chitin (Sigma-Aldrich, USA) were acid hydrolyzed by slowly adding 300 ml of concentrated HCl with continuous stirring for one hour. Thereafter, the acid hydrolyzed mixture was filtered using cheesecloths, and the partially hydrolyzed chitin was suspended in 1 L of cold distilled water and was left overnight at 4°C. The colloidal chitin suspension was collected by filtration using coffee filter paper and neutralized with 1 N of cold NaOH solution followed by washing several times with cold distilled water. The neutralized colloidal chitin was stored at 4°C until date of use.

2.2. Extracellular Crude Chitinase Production in Submerged Culture

The bacterial isolate A7 was cultivated in l L Erlenmeyer flask containing 500 mL of liquid chitin medium on an orbital shaker (150 rpm, Forma Orbital Shaker, Thermo Fisher Scientific, USA) at 27°C for eight days. During the fermentation process, 5 mL sample and thereafter daily samples were taken to monitor the bacterial growth and to estimate chitinase activity. The growth was followed by optical density (OD) measurements of 10-fold diluted samples at 580 nm (UV/Vis Spectrometer, Lambda 16, Perkin Elmer, Germany), changes in pH value (pH 523, WTW, Germany) and measurement of chitinolytic activity of the cell free culture. As the OD started to decrease or the maximum activity was attained, the cultivation process was terminated and the culture fluid was separated from the bacterial cells by centrifugation (5,000 g for fifteen minutes, Beckman GS-6, USA). Chitinase activity in the culture filtrate was estimated using the Dinitrosalicylic acid method (DNS).

2.3. Identification of B. atrophaeus A7

Bacillus atrophaeus A7 was characterized morphologically (colony form and appearance, cell shape and size) and biochemically (Gram stain, oxidase, catalase, nitrate reduction, sugar utilization, methyl red and indole tests) for 48-72 hours grown colonies on nutrient agar plates. The biochemical characteristics were determined using standard procedures (Collins *et al*., 2004; York *et al*., 2007). The production of acid from different carbohydrates was detected as described by Helmke and Weyland (1984). Moreover, the identification of the isolate was confirmed by 16S rDNA gene sequencing as described previously (Al-Zereini *et al*., 2007).

The 16S rDNA gene sequence was compared with those available in the GenBank and Ribosomal database project II (RDP). Molecular phylogenetic analysis was conducted using MEGA7 software (Kumar *et al*., 2016). Evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model (Tamura and Nei, 1993).

2.4. Chitinase Activity Assay

The activity of the crude chitinase enzyme was determined using standard DNS method (Dixit *et al*., 2015). Briefly, 1 mL of the culture supernatant containing the crude enzyme was added to 1 mL of 1% (w/v) colloidal chitin solution prepared in 50 mM acetate buffer (pH 4.8). The assay mixture was incubated in a water bath at 37°C for thirty minutes, and the reaction was stopped by adding 1 mL of DNS reagent with boiling for ten minutes. Thereafter, the mixture was left to cool, then centrifuged (5,000 g for fifteen minutes), and the amount of the reducing sugar (N-acetyl-D-glucosamine) released was measured spectrophotometrically at 575 nm. One unit of the chitinase enzyme was defined as the amount of enzyme that produced 1 µmol of N-acetyl-D-glucosamine/min from 1 % (w/v) colloidal chitin solution prepared in 50 mM acetate buffer (pH 4.8) at 37 °C (Miller, 1959).

As a blank, 1 mL of boiled inactive enzyme was added to 1 mL of 1 % colloidal chitin under the same experimental conditions. A standard curve was constructed

by measuring the color intensity of 1 ml of N-acetyl-D-glucosamine solutions (1, 2, 5, 8 and 10 mM) dissolved in 50 mM acetate buffer (pH 4.8). The total protein content in the crude enzyme supernatant was determined using Lowry method (Lowry *et al*., 1951) with bovine serum albumin as standard.

2.5. Effect of Temperature and pH on Chitinase Activity and Stability

Determination of the optimum temperature for the chitinase activity was carried out by standard DNS assay with incubation at different temperatures (20ºC to 60ºC) with 10ºC temperature intervals. The thermostability of the chitinase enzyme was determined by pre-incubating the crude enzyme supernatant in 50 mM acetate buffer (pH 4.8) at different temperatures (30ºC to 80ºC) with 10ºC temperature intervals for one hour. The residual activity was determined using 1 % (w/v) colloidal chitin as substrate at standard assay conditions (50 mM acetate buffer, pH 4.8 at 37^{o}C and stopping the reaction by adding 1 mL of DNS reagent with boiling for ten minutes).

The optimal pH for chitinase activity was determined at 37^{o}C and different pH values (pH 3-10) with 1 pH intervals by standard DNS assay using 1 % (w/v) colloidal chitin as substrate. The solutions used were 50 mM of either citrate buffer (pH 3-6) or phosphate buffer (7-8) or Tris-base (pH 8-10). The effect of pH on the enzyme stability was evaluated by pre-incubating the crude enzyme supernatant at 37^{o}C for one hour in the same buffer ranges. The residual enzyme activity was determined under the standard assay conditions using 1 % (w/v) colloidal chitin as substrate.

2.6. Effect of Metal ions on Chitinase Activity

As several enzymes may require certain metal ions as a component of the active site to maintain its stability and for their catalytic activity, the effect of various metals on the chitinase activity was studied by performing the standard assay using 1 % (w/v) colloidal chitin with different metal ions ($CuSO_4$, $FeCl_3$, $NiCl_3$, $ZnCl_2$ and $BaCl_2$) at a final concentration of 10 mM at 37ºC. The relative inhibition in the enzyme activity was based on measuring the released N-acetyl-D-glucosamine.

2.7. Larvicidal Activity of Bacillus Atrophaeus A7 Chitinase on the Development of Drosophila Melanogaster 2nd Instar Larvae and Determination of the Lethal Dose (LD50)

Half gram of the blue colored instant *Drosophila* media (Formula 4-24®, Carolina Biological Supply Company, USA) was soaked in 2 mL of diluted crude chitinase supernatant in final concentrations ranging from 0 mU/mL up to 68.9 mU/mL. As control, 2 mL of the diluted heat-inactivated crude enzyme supernatant (at 100°C for thirty minutes) was used at final concentrations equivalent to the active crude enzyme. The media in the vials were left for few minutes to gel, and were then overlaid with 10 of *D. melanogaster* 2nd instar larvae.

The required concentration of the chitinolytic crude supernatants to cause death of 50 % (LD_{50}) of *D. melanogaster* larvae was determined under controlled laboratory conditions (21-22^{o}C, germ-free medium throughout the test) as recommended in the working sheet of Formula 4-24® medium by Carolina Biological Supply Company. The effect of different concentrations of the chitinolytic crude extract of *B. atrophaeus* A7 on the development of the larvae into pupal and adult stages was observed daily (loss of larval intensive activity as voracious feeders and foraging ability) for a period of eight days in both the control and experimental treatments. The dose-response relation was appraised using PROBIT regression analysis with 95 % confidence limit (Finney, 1978).

To confirm that the larvicidal effect is mainly attributed to the activity of the crude chitinase of *B. atrophaeus* A7, the chitinase inhibitor pentoxifylline (Sigma-Aldrich, USA) was added to 10 ml of the crude enzyme supernatants to a final concentration of 250 and 500 µg/mL. The preparations were left for thirty minutes at 37^{o}C, after which 2 mL were withdrawn and added to the insect medium; the medium was overlaid with 10 *D. melanogaster* 2nd instar larvae and was monitored daily for larval development.

2.8. Statistical Analysis

Data were represented as mean averages of three independent assays with standard deviations. Mean values and standard deviations as well as PROBIT analysis were deduced using *Microsoft Excel software*. Results were analyzed by one-way ANOVA and Tukey HSD post hoc test using the Statistical Package for the Social Sciences software (SPSS, version 16). Data were considered significant at $p<0.05$ (one-way ANOVA). Tukey HSD post hoc test was carried out on data where $p<0.01$ to identify which of tested factor pairs are significantly different from each other.

3. Results

3.1. Bacterial Isolate Identification and Crude Chitinase Activity Measurement.

Bacterial isolate A7 is a Gram-positive spore forming bacillus, forms circular colonies of 1.5-2 mm in diameter and of a white opaque colour with undulating margins. It is oxidase-negative, catalase-positive bacterium, able to utilize glucose, sucrose, and mannitol, but not maltose, xylose, or lactose as a carbon source, reducing nitrate and hydrolyzes gelatin. It gave positive results in indole and methyl red tests, and was identified morphologically and biochemically as a member of the genus *Bacillus*. 16S rRNA sequencing indicated that isolate A7 is related to *B. atrophaeus* (Accession no. KU 955655.1) with a similarity level of 99 % (Figure 1). A photograph for the bacterial isolate A7 colonies grown on chitin agar and showing its ability to produce extracellular chitinase is presented (Figure 2).

The production of chitinase was detected after twenty-four hours of *B. atrophaeus* cultivation in a liquid chitin medium with approximating the maximum activity at the 8th day of growth. The total activity measured by DNS assay was 68.9±1.03 mU/mL with a specific activity of 2670±40.2 mU/mg. The increase in the daily production and subsequently the chitinolytic activity of the exochitinase is illustrated (Figure 3). However, prolonged cultivation time resulted in a reduction and even loss of the chitinase activity.

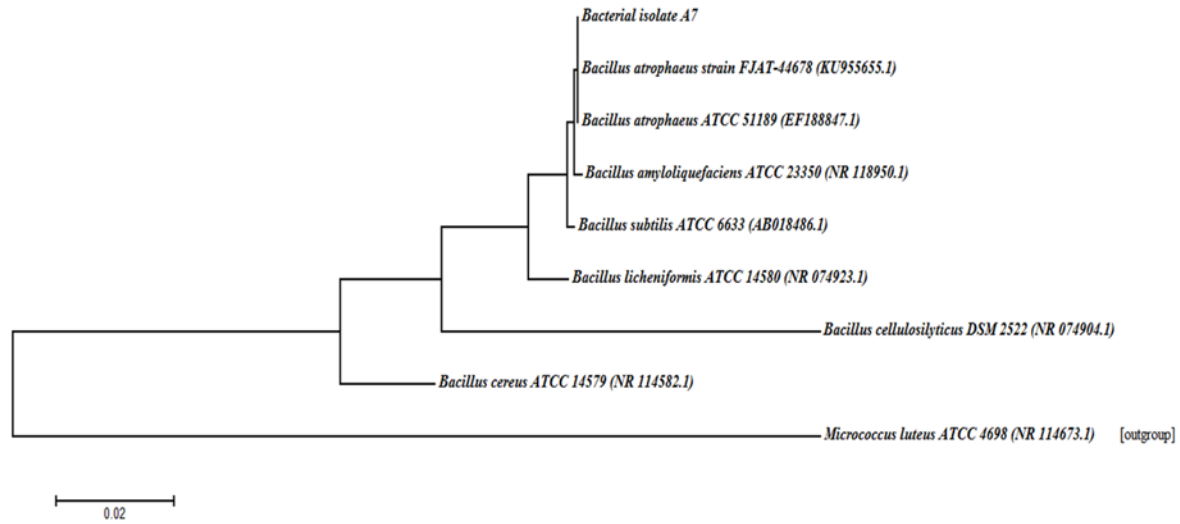

Figure 1. Phylogenetic tree of bacterial isolate A7, constructed by Maximum Likelihood Algorithm using MEGA7 software. The scale bar indicates 2 % estimated base substitution per nucleotide position.

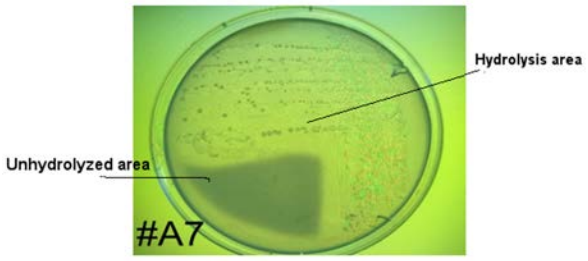

Figure 2. Hydrolysis zone formed during the growth of bacterial isolate A7 on a chitin agar plate incubated at 37°C for seven days. The wide clear areas around the gray colonies grown on the plate indicate the hydrolysis of chitin.

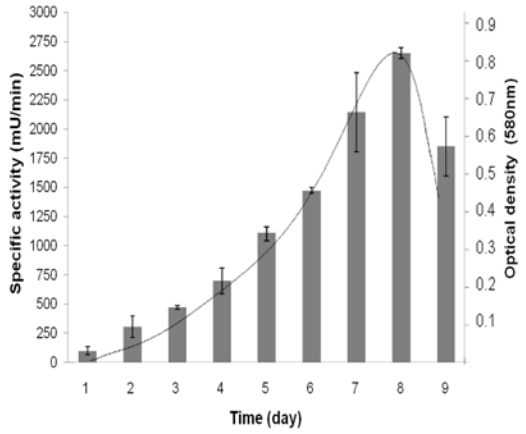

Figure 3. Chitinolytic activity of daily samples collected during the growth of *B. atrophaeus* A7 in a chitin liquid medium at pH 7.2 and 27 °C.

3.2. Effect of Temperature and pH on Crude Chitinase Enzyme Activity and Stability

Under the experimental conditions, the optimum enzyme activity was achieved at 40°C with a decrease in the relative activity to 81±11.1% (55.8±7.6 mU/mL) at 60°C. Nevertheless, data analysis indicated that carrying out the enzyme assays at different tested temperatures did not significantly affect the enzyme activity (p>0.05). The crude enzyme was stable, so the optimum activity was maintained for one hour at 30-40°C; however, this activity was significantly altered at temperatures above 40°C (p<0.05). In fact, a maximum loss of the enzyme activity was noticed after one hour at 80°C (42±4.2%, 40±2.9 mU/mL) (Figure 4 A and B).

The chitinolytic enzyme produced by *B. atrophaeues* was active with insignificant differences in its relative activity at a broad pH of 4-9 (p>0.05). Intriguingly, significant reduction in the total activity to 80 % (55.1±2.76 mU/mL) and 85 % deactivation (10.34±2.55 mU/mL) was detected at 10 and 3 pH, respectively (p<0.01). However, the enzyme was stable at 6-7 pH with a significant reduction in its activity to 40 %-50 % at 8-10 or 4-5 pH and 95 % at pH 3 (Figure 4 C and D).

3.3. Effect of Different Metal Ions on Crude Chitinase Enzyme Activity

The activity of chitinase from *B. atrophaeus* A7 was highly inhibited with 10 mM of all used metal ions (p < 0.01). In fact, the order of potency of the tested metal ions to inhibit the chitinase activity was Fe^{3+} > Ni^{3+} > Cu^{2+} > Zn^{2+} > Ba^{2+}. Iron ions (Fe^{3+}) caused 90 % inhibition in the enzyme activity under the assay conditions; meanwhile 60-75 % inhibition was obtained by other metal ions (Figure 5).

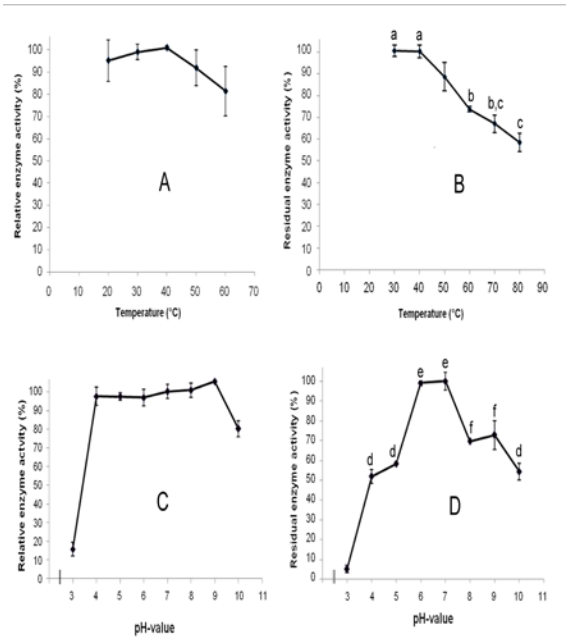

Figure 4. Effect of different temperatures and pH values on activity and stability of crude chitinase from *B. atrophaeus* A7. A) relative enzyme activity at different temperatures for thirty minutes, pH 4.8; B) residual activity as a measure of enzyme stability at different temperatures for one hour, pH 4.8; C) relative enzyme activity at different pH values for thirty minutes, 37°C; and D) residual activity as a measure of enzyme stability at different pH values for one hour, 37°C. Means with similar letters are not significantly different from each other based on post hoc Tukey HSD test at $p<0.01$.

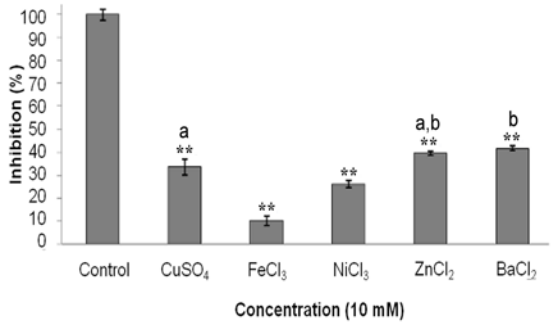

Figure 5. Effect of different metal ions on the activity of the chitinase enzyme from *B. atrophaeues* A7 at 10 mM. Asterisks indicated statistically significant values from control (** p<0.01). Means with similar letters are not significantly different from each other based on post hoc Tukey HSD test at $p<0.01$.

3.4. Larvicidal Activity of Crude Chitinase Enzyme from B. atrophaeus A7 on D. Melanogaster and Determination of LD50

The Chitinolytic enzyme produced by *B. atrophaeus* A7 interfered with the development of *D. melanogaster* larvae. More than 80 % of these larvae were unable to metamorphose into the 3rd instar stage, pupae, or emerge as adult flies at 27.56 mU/mL. Plotting the dose-larval response to the crude chitinase enzyme (Figure 6) and the PROBIT analysis calculated from the bioassay 8th day post-treatment revealed that LD_{50} is 17.3±0.9 mU/mL

. The lethal effect of the chitinase was concentration-dependent, and the larvae development was significantly retarded at most applied concentrations as compared to control ($p<0.01$).

The development of 60-70 % of larvae to adults was delayed to the 6th day under the experimental condition at 13.8 mU/mL and the addition of pentoxifylline caused 1.4 (24.7±1.2 mU/mL) and 1.8 (31.7±1.5 mU/mL) fold increase in LD_{50} at 250 and 500 µg/ml, respectively.

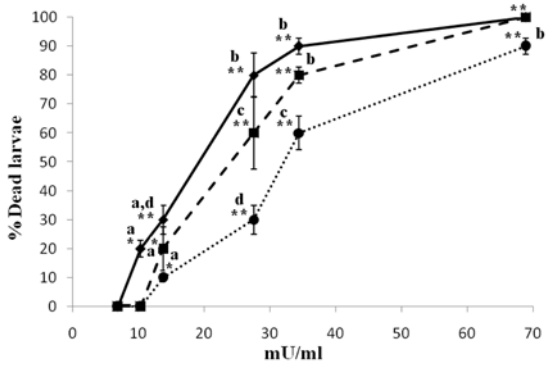

Figure 6. Effect of the crude chitinase enzyme from *B. atrophaeues* A7 on survival and development of the 2nd instar *D. melanogaster* larvae to adults. Asterisks indicated statistically significant values from control treated with heat inactivated-crude chitinase (*: 0.01<p<0.05, ** $p<0.01$). The percentage of the dead larvae means with similar letters are not significantly different from each other based on post hoc Tukey HSD test at $p<0.01$. Solid line: chitinase without pentoxifylline; dash line: chitinase with 250 µg/mL pentoxifylline; and dots: chitinase with 500 µg/mL pentoxifylline.

4. Discussion

Despite the fact that chitinases play a role in the arthropod insects' molting process (edysis), since their exoskeleton and gut lining consist largely of chitin, it was postulated that the bacterial chitinase could be involved in biocontrol regimes aiming to select potential insecticidal agents. Bacterial chitinase plays a role not only in recycling chitin in nature to provide carbon and nitrogen as a nutrient, but also a role in plant defense systems against chitin-containing pathogens and biocontrol (Zhu *et al*., 2007). The production of more than one type as well as multiple forms of chitinases was reported in different *Bacillus* spp. such as *B. amyloliquefaciens* and *B. megatrium* (Sabry, 1992), *B. cereus* (Chang *et al*., 2007), *B. licheniformis* (Waldeck *et al*., 2006), and *B. subtilis* (Wang *et al*., 2006). Nevertheless, the chitinase production and activity from *B. atrophaeus* were not utterly contemplated.

In the current study, the production of the chitinolytic enzyme from *B. atrophaeus* A7 was observed since the first day following the bacterial cultivation under experimental conditions; however, the decrease in activity with a prolonged growth regime after the 8th day might be due to the reduced level of nutrients in the culture medium and/or denaturation of chitinase by proteases (Zarei *et al*., 2010). The optimum enzyme activity and stability of the isolated chitinase from *B. atrophaeus* A7 at different temperatures and pH values coincided with previously reported bacterial chitinases. Previous studies showed that the chitinase produced by *B. atrophaeus* has an optimum working temperature of 50ºC and optimum pH of 8, while

that from *B. subtilis* was active at 40ºC and pH 5. Moreover, chitinase from both strains was still active up to pH 8 (Anuradha and Revathi, 2013). The chitinolytic enzyme from *Bacillus* sp. WY22 was optimally active at 37ºC and pH 5.5, and was stable at 30-40ºC and pH 4.5 (Woo and Park, 2003). *B. laterosporus* MML2270 produced chitinase with the highest activity at 35ºC and pH 8.0, and interestingly, it was inactive at pH 4 (Shanmugaiah *et al*., 2008).

Several bacterial chitinases have broad pH stability, and they are thermotolerant. Chitinases from *B. licheniformis* have optimum temperature at 60-65ºC and optimum pH 6, and were stable up to 60ºC and broad pH 4-11 (Sandalli *et al*., 2008; Songsiriritthigul *et al*., 2010). *B. cereus* TKU 028 produced chitinase with an optimum temperature of 60ºC and pH 5, and was stable for sixty minutes at temperatures below 60ºC and over a broad pH range from 4 to 9 (Liang *et al*., 2014). The chitinase from *Bacillus* sp. 7079 was optimally active at pH 7.5 and 45ºC; stable under wide pH range between pH 5 and 9, and more than 80% of the original activity was retained at 40ºC (Kyung *et al*., 2003). Other bacterial chitinases were maximally-active at 50ºC and pH 8 with a stability at 50ºC and 6-9 pH in *Pseudomonas aerogenosa* (Hiraga *et al*., 1997); at 40ºC and 5-8 pH in *Aeromonas hydrophila* H-2330 (Wang and Chang, 1997); at 40ºC and pH 7.5 in *Pseudoalteromonas* sp. DXK012 (Liu *et al*., 2015); and at 30ºC and pH 5 with stability at 40ºC and pH 8 in *Streptomyces* sp. M-20 (Kim *et al*., 2003).

The stability of bacterial chitinase at different conditions, including broad pH ranges or temperatures, refers to the their production of more than one isoform of the enzyme (Kuzu *et al*., 2012), or this is because of the deletion or insertion of additional domains at both N- and C-terminals that keep its structure more stable, and thus avoiding its disturbance in these conditions (Chen *et al*., 2015; Thimoteo *et al*., 2017). Two chitinase isoforms with a molecular mass 43 and 18.5 KDa were reported from fluorescent pseudomonads with antifungal activity (Ajit *et al*., 2006). Six isoforms (39-92 KDa) were produced by *Streptomyces* S242 (Saadoun *et al*., 2009). Six chitinases were reported from *B. circulans* and *Bacillus* sp. with the molecular mass 38-73 KDa and 22–77 KDa, respectively (Watanabe *et al*., 1990; Sakai *et al*., 1998).

The activities of most enzymes, if not all, require and are affected by the presence of certain metal ions. Herein, all examined metals significantly inhibited the activity of the chitinolytic enzyme produced by *B. atrophaeus*, and the extent of inhibition was metal ion type dependent. The findings of the current study agreed with previous reports on bacterial chitinases, wherein they were inhibited at 1 mM of different divalent metals (Sri *et al*., 2004; Lee *et al*., 2007; Annamalai *et al*., 2010; Zarei *et al*., 2011; Cheba *et al*., 2016). At 1 mM concentration, Cu^{2+}, Fe^{3+}, Ni^{3+}, Zn^{2+} and Ba^{2+} offered partial inhibition in the chitinase activity of *Serratia marcescens* B4A (Cheba *et al*., 2016) and *Bacillus* sp. R2 (Zarei *et al*., 2011). In contrary to the results of this study, it was reported that chitinase was activated by 10 mM of $ZnSO_4$ in *B. atrophaeus* (Anuradha and Revathi, 2013); by 5 mM of $FeSO_4$ and $NiCl_2$ in *Micrococcus* sp. AG84 (Annamalai *et al*., 2010), while it was not affected by 1 mM of $BaCl_2$ in *Alcaligenes xylosoxydans* or *Serratia marscesens* B4A (Vaidya *et al*., 2003; Zarei *et al*., 2011) indicating that the bioactive site of the herein isolated chitinase most probably structurally different from those tested in the above studies. The role of such metal ions in the enzyme inhibition may be attributed to the ability of these ions to attach the catalytically active subunits of the enzyme by forming complexes with carboxylic groups of aspartic and glutamic acid residues at the active site, changing the conformational shape of the enzyme and competing with cation activators bound with the formation of a substrate-enzyme complex (Donderski and Brzezinska, 2005; Thimoteo *et al*., 2017).

In developing countries, land cultivation represents part of the main individual's income and living needs. Consequently, the control of plant pests and diseases-related insect vectors is crucial to the reliable production of food and healthcare management. However, the living and breeding of *D. melanogaster* in high-density, and within microbe-rich food substrates such as rotten and decaying fruit endowed its evolutionary adaptation to interact naturally with pathogenic bacteria (Keesey *et al*., 2017). In fact, it could be exploited by these bacteria as a potential vector between plants accounting, even more, for a possible threat to human health. Therefore, the exploring of an easily-handled natural source, such as these microorganisms, for environmental-friendly insecticides against fruit fly larvae was of high priority.

To date, chitinases with the insecticidal action were known from *B. thuringiensis*, *B. cereus*, *B. subtilis* (Liu *et al*., 2002; Chandrasekaran *et al*., 2012) and several actinomycete species (Gadelhak *et al*., 2005). *Bacillus thuringiensis* is a well-known biocontrol agent that has been in use for decades for pest control in agriculture and for the control of disease-related insect vectors (Veliz *et al*., 2017). Its toxins had been demonstrated against many typical insect orders, such as Lepidoptera (butterflies and moths), Diptera (flies and mosquitoes) and Coleoptera (beetles and weevils) (Xu, 2014) that encompass major invertebrate plant pests from agricultural as well as some insect vectors of mammalian pathogens (Nester *et al*., 2002). The activities of these toxins were attributed to the parasporal crystals (Cry proteins), which are produced and assembled during *B thuringiensis* sporulation. After ingestion by larvae, the crystals are solubilized and converted into active toxins by proteases in the larval midgut causing the death of the larvae. Meanwhile, chitinase from different actinomycetes played an important role against the house fly *Musca domestica* and is used against *Culex quinquefasciatus* (Gadelhak, 2005).

Herein, the larvicidal activity of *B. atrophaeus* A7 is reported against the development of the *D. melanogaster* 2nd instar larvae into adult fruit fly. Although other potential effectors, such as proteases and toxins, could be one of the fundamental causes of the larvicidal activity. However, the inability of the larvae to ecdyse and develop to 3rd instar larval and hereafter to pupal stages hint for cuticle defect due to the chitinolytic attack. Moreover, the increase in crude enzyme concentration that cause 50 % inhibition in the larval development (LD_{50}) in presence of the chitinase inhibitor pentoxifylline indicated that such larvicidal activity is attributed, mostly if not entirely, to the effect of the tested crude chitinase supernatant.

The current findings coincided with the reported activity of chitinases from both *Actinomyces phlippinensis* and *A. missouriensis* that had significantly reduced *D. melanogaster* pupal formation when applied to the rearing medium individually (Gadelhak 2005). Chitinolytic enzymes may inhibit chitin formation either by affecting the catalytic site of the synthase, by interfering at the sulfhydril-sensitive sites of the synthase during polymerization of the β-1,4-N-acetyl-D-glucosamine residues, or by the ability to interfere with chitin deposition (Gadelhak *et al*., 2005). Besides, chitinase applied in an insect's rearing medium induces damage to the peritrophic membrane in the insect gut causing a significant reduction in the nutrient utilization, and consequently affecting insect development and growth (Deepthi, 2014).

Interestingly, it was documented that the chitinase-producing *B. thuringiensis* enhanced the insecticidal toxicity of the active *B. thuringiensis* against newly-hatched beet armyworm *Spodoptera exigua* larvae with an enhancing ratio of 2.35-fold (Liu *et al.,* 2002). Hence, *B. atrophaeus* A7 might be a source of insecticidal chitinase that could act individually as a biopesticide agent or in synergistic with other bioinsicticides by enhancing their effectiveness.

5. Conclusion

This study represents the first report that verifies the ability of *B. atrophaeus* to produce chitinolytic enzyme with a potential insecticidal activity. The produced chitinase was active at broad pH ranges, and was thermotolerant suggesting that it could be useful for biotechnological and agricultural applications. Further work is in plan for the molecular characterization of chitinase genes, to purify and determine the type and molecular weight of chitinase produced by *B. atrophaeus* A7.

Conflict of Interest

The authors declare that they have no conflict of interest.

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JJBS

Jordan Journal of Biological Sciences

Volume 11, Number 4, September 2018
ISSN 1995-6673
Pages 461 - 467

Bisphenol-A Hepatotoxicity and the Protective Role of Sesame Oil in Male Mice

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Received January 23, 2017; Revised February 20, 2018; Accepted February 27, 2018

Abstract:

The present study is conducted to estimate the protective effects of sesame oil (SO) against bisphenol-A (BPA) induced hepatotoxicity in male mice. BPA (50 mg/kg b wt) was gavages orally to mice once a day for twenty-eight successive days. While, SO was administered to the mice orally at 1 mL/kg b wt. through three regimens (before, with or after) treatment of BPA. After the complementation of treatments, the liver of each sacrificed animal was subject to DNA damage evaluation using alkaline comet assay and histopathological examination. In addition, blood was collected for biochemical assessment of the liver function. Results showed that, BPA significantly reduced the relative liver weight, and caused DNA damage, several histopathological alterations in liver tissue and elevation in ALT and AST compared to the control. On the contrary, oral gavages of SO with BPA was effective in the increase of relative liver weight, amelioration of DNA damage and histopathological alterations, and in the reduction of the liver enzyme levels compared to BPA's treated mice. Furthermore, the best alleviation of the undesirable effects of BPA appears in the administration of SO to mice before the BPA treatment, followed by after treatment and during or with BPA treatment. In conclusion, the present study provided clear evidence that SO possesses a promising protective activity against the noxious effects of BPA.

Keywords: Bisphenol-A, Hepatotoxicity, Sesame oil, DNA damage, Histopathological alterations.

1. Introduction

Bisphenol-A (BPA) is a famous endocrine disruptor fabricated compound (Michalowicz, 2014) widely utilized in the manufacturing of polycarbonates, epoxy resins and thermal paper (Hoekstra and Simoneau, 2013), food and beverage containers, utensils, baby tools, protective coatings and CD (Maffini *et al.*, 2006; Biedermann *et al.*, 2010).

A huge quantity of BPA is continuously released into the environment through different routes, including sewage, landfill leach and domestic waste combustion (Gassara *et al.*, 2013). Therefore, BPA has become ever-present in the environment (Flint *et al.*, 2012). Healthwise, BPA causes hepatotoxic, mutagenic, reproductive and carcinogenic effects (Doherty *et al.*, 2010; Erler and Novak, 2010; Meeker *et al.*, 2010; Zeinab *et al.*, 2012). Moreover, Lee *et al.* (2003) stated that BPA induced DNA strand-breaks in mouse lymphoma cells. In addition, it can induce liver, kidney, brain, and other organ injuries by generating reactive oxygen species (ROS) (Bindhumol *et al.*, 2003; Kabuto *et al.*, 2004).

Sesame oil (SO) is obtained from the *Sesamum indicum* L. seeds that belong to the family of Pedaliaceae. It contains many fatty acids and antioxidants, including sesamin, sesamolin, sesamol, and tocopherol, (Fukuda, 1990). Furthermore, it offers lipid peroxidation, the best protection by increasing nonenzymatic and enzymatic antioxidants (Sankar *et al.*, 2005). Many studies revealed that SO attenuated the hepatotoxicity induced by numerous compounds in experimental animals (Erol *et al.*, 2011; Srinivasan and Liu, 2012; Periasamy *et al.*, 2012; 2014; Azab, 2014; Soliman *et al.*, 2015). Whereas, Cengiz *et al.* (2013) reported that SO treatment was not significantly effective in the mitigation of histopathological disturbances in CCl_4 treated rat liver. Therefore, the present research is designed to evaluate the protective effects of SO against the hepatotoxicity induced by BPA in male mice.

2. Materials and Methods

2.1. Chemicals

Bisphenol-A (BPA) (≥ 99 %) was purchased from Sigma-Aldrich Company (St. Louis, MO, USA). Commercial sesame oil was purchased from EL Captin

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Company (Al Obour City, Cairo, Egypt). The aspartate aminotransferase (AST) and alanine aminotransferase (ALT) kits were purchased from Biodiagnostic co., Egypt. All other chemicals were of analytical grade and were purchased from standard commercial suppliers.

2.2. Dose Preparation

BPA was dissolved in absolute ethyl alcohol (95 %) and diluted with corn oil [1:20 alcohol: corn oil (vehicle)] to obtain a final concentration of BPA (50 mg/kg b wt). It was freshly prepared before use.

2.3. Animals and Treatments

Male Swiss albino mice weighing 26±5 g (10-12 week old) were purchased from Theodor Bilharz Research Institute, Giza, Egypt. They were housed in polypropylene cages (43×30×15cm, 5 mice per cage) with stainless steel covers in the Animal House of Environment and Bioagriculture Department, Faculty of Agriculture, Al-Azhar University. The animals were kept under controlled temperature (23±4 °C), 50–55 % relative humidity, and a photoperiod of 12 hours of light: 12 hours of dark cycle. After acclimation for two weeks, the animals were randomly divided into seven groups (n = 10) with approximately equal mean body weights. Animals were administered orally for twenty-eight successive days for BPA (50 mg/kg b wt) and/or SO (1mL/kg b wt) either before, with, or after the BPA administration as follows: group one (control); group two (vehicle); group three (SO); group four (BPA); group five (SO before BPA); group six (SO with BPA) and group seven (SO after BPA).

2.4. Oil Analysis

2.4.1. Fatty Acids Analysis

The fatty acid profile was determined as fatty acid methyl esters by Thermo scientific TRACE 1310 gas chromatograph attached with ISQ LT single quadrupole mass spectrometer (GC-MS). The preparation of the fatty acid methyl esters (FAMEs) was performed according to the procedure of AOAC (2000). A sample of the oils (50 mg) and 1 % H_2SO_4 in absolute methanol were put in screw-cap vial (2 mL). The vial was covered under a stream of nitrogen before being heated in an oven at 90 ^oC. Finally, 1µL of the solution obtained was injected into the GC–MS system after the addition of the internal standard. The fatty acid methyl esters (FAMEs) composition analysis was performed in an Thermo scientific TRACE 1310 gas chromatograph attached with ISQ LT single quadrupole mass spectrometer with DB1, 15m; 0.25mm ID (J&W scientific) capillary column. Helium was used as a carrier gas with a flow rate of 1.5 mL min^{-1}, and the injector temperature was maintained at 200^o C. the oven temperature was programmed with an initial temperature of 115^o C for one minute and was then increased to 280^o C by 7.5^o C min^{-1} for three minutes.

2.4.2. Total Phenolic Content

The total phenolic content (TPC) was determined using the Folin–Ciocalteau's reagent according to the method reported by Lin and Tang (2007) at 760 nm with spectrophotometer (UV-1601; Shimadzu, Tokyo, Japan. The quantification was done on the basis of the standard curve of gallic acid concentration ranging between 10 to 80 mg/mL (r^2= 0.99).

2.4.3. Determination of the Total Tocopherol (Vitamin E)

High performance liquid chromatography (HPLC) system (1100 series, Agilent Technologies) was used for the quantification of vitamin E based on a method described by Gimeno *et al.* (2000). The oil sample was diluted in hexane (1:10). Thereafter, 200 µL was transferred to a screw-capped tube, where 600 ml of methanol and 200 mL of the internal standard solution (300 µg/mL of α-tocopherol acetate in ethanol) were added. After being vortex-mixed and centrifuged (3000 g, 5 min), the samples were filtered through a 0.45 mm pore size filter, and an aliquot of the overlay was directly injected into the chromatograph.

2.4.4. Measurement of the Antioxidant Activity

The ability of SO at 200 uL to scavenge 2.9 mL of 1, 1'-diphenyl 1-2-picrylhydrazyl (DPPH) free radical was estimated by the method of Singh *et al.* (2002).

2.5. Relative Liver Weight

At the termination of the experiments, livers were dissected out, trimmed of excess fat and weighed before the genetic and histopathological studies took place. The liver weight was presented as relative organ weight as follows:

$$\text{Relative organ weight} = \frac{\text{Absolute organ weight (g)}}{\text{Final body weight (g)}} \times 100$$

2.6. Evaluation of DNA Damage

2.6.1. Comet Assay in the Liver Cells

The comet assay was performed in the liver cells according to Bandyopadhyaya *et al.* (2008). Briefly, 50 µL of cell suspension was mixed with 100 µL of 1 % low melting point (LMP) agarose and were added to fully-frosted slides coated with 80 µL of 1 % normal melting point (NMP) agarose. The cells were then incubated in a lysis solution (2.5 mol L^{-1} NaCl, 100 mmol L^{-1} EDTA, 10 mmol L^{-1} Tris-HCL, 1 % Triton X-100, pH 10) at 4 °C for at least two hours, at which the slides were placed into an alkaline solution (300 mmol L^{-1} NaOH, 1 mmol L^{-1} EDTA, pH 13) at 4 °C for twenty minutes to allow for the DNA unwinding, and were electrophoresed at 25 V (300 mA) for twenty minutes . Finally, the slides were neutralized in a 400 mmol L^{-1} Tris buffer (pH 7.5) for fifteen minutes and were stained with ethidium bromide (5 µg mL^{-1}). Images of fifty randomly selected nuclei per experimental group were captured using a fluorescence microscope (Eclipse 800, Nikon, Tokyo, Japan). They were analyzed with image analysis software (Comet Assay IV, Perceptive Instruments, Suffolk, UK). The scored parameters included the tail length, DNA percentage in tail and olive tail moment (OTM). Tail length is the maximum distance that the damaged DNA migrates from the center of the cell nucleus. The percentage of DNA in Tail is the DNA content that migrates from the nucleus into the comet tail. OTM is the product of the tail length and percentage of DNA, which gives a more integrated measurement of the overall DNA damage in the cell.

2.7. Histopathological Examination

Liver from each sacrificed mouse was dissected out and trimmed of excess fat tissues. The tissues were fixed in 10 % buffered formalin and were processed for paraffin sectioning by dehydration in different concentrations of alcohol, cleared with xylol, and embedded in paraffin blocks. Sections of about 5µm thick were stained with Harris haematoxylin and eosin (H&E) for a histological study (Delafield, 1984).

2.8. Biochemical Analysis

Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) liver enzyme activities were measured in serum by the method of Reitman and Frankel (1957).

2.9. Statistical Analyses

Statistical analyses were performed with SPSS 16 software. The experimental data were analyzed using one-way analysis of variance (ANOVA). The Duncan's multiple range test was used to determine the significant differences between means. All values were expressed as mean ± SD, and the significance level was set at $P \leq 0.05$.

3. Results and Discussion

3.1. Sesame Oil Analyses

3.1.1. Fatty Acids

The fatty acid composition is an essential indicator of the dietary nutritional value of the oil. As summarized in Table 1, the main fatty acids in SO are palmitic (13.57±2.71), stearic (4.23±0.41), oleic (42.93±3.76) and linoleic (35.77±2.21) in addition to some traces of palmitoleic (0.67±0.25), heptadecanoic (0.43±0.20), linolenic acid (0.73±0.40), arachidic (0.87±0.20) and eicosenoic (0.77±0.15). Hassan (2012) revealed that sesame oil belongs to the oleic–linoleic acid group. It has less than 20 % saturated fatty acid consisting mainly of palmitic and stearic acids. Oleic acid and linoleic acid constitute more than 80 % of the total fatty acids in the sesame seed oil. It has been mentioned that edible oil fatty acids may exert beneficial health effects by the modulation of signaling pathways regulating cell differentiation and proliferation (Lewinska *et al*., 2015).

3.1.2. Phenolic and Vitamin E Content

Sesame seeds are a rich source of antioxidants and bioactive compounds including phenolics. In addition, among the vitamins of sesame seeds, vitamin E is very essential which makes sesame seeds efficient as healthy food (Pathak *et al.*, 2014). The analysis of sesame oil revealed that SO's phenolic and vitamin E content was (15±2.0) and (6.77±0.77), respectively as shown in Table 1.

3.1.3. Antioxidant Activity

The antioxidant activity measurement showed that sesame oil at 200 µL exhibited an antioxidant activity of 61.03±0.74 as shown in Table 1. Previous studies revealed that the sesame oil possesses a strong antioxidant activity that greatly boosted its applications in the healthy food products because of its efficiency in the protection of the liver and the heart. In fact, sesame oil can be classified as edible oil with a high potential of antioxidant activity (Cheng et al., 2006; Bopitiya and Madhujith, 2013).

Table 1. Chemical composition and antioxidant activity of sesame oil.

Parameters	**Value**
Fatty acids	**(%)**
Palmitic	13.57±2.71^{c}
Palmitoleic	0.67±0.25^{de}
Heptadecanoic	0.43±0.20^{de}
Stearic	4.23±0.41^{d}
Oleic	42.93±3.76^{a}
Linoleic	35.77±2.21^{b}
Linolenic	0.73±0.40^{de}
Arachidic	0.87±0.20^{de}
Eicosenoic	0.77±0.15^{de}
Total phenolic content (mg gallic acid /100g oil)	15.00±2.00
Vitamin E concentration (µg/mL)	6.77±0.77
Percentage of antioxidant activity (200 uL)	61.03±0.74

Data are expressed as means ± SD. Mean values in the same column within each parameter bearing the same superscript do not differ significantly ($P \leq 0.05$).

3.2. Relative Liver Weight

3.2.1. Effects of BPA on Relative Liver Weight

According to the data in Table 2, the oral administration of BPA to male mice at a dose of 50 mg/kg b wt for twenty-eight consecutive days caused a significant decrease ($p \leq 0.05$) in the relative liver weight (3.19±0.05) compared to the control (3.38±0.05). These results are in agreement with the findings of Hassan *et al.* (2012) who demonstrated that the oral administration of BPA to male rats at the dose of 0.1 mg/kg/day for four weeks induced a significant decrease in the body weight compared to the control. In addition, previous data demonstrating that exposure to BPA during embryo development and infancy decreased the organ weights of male mice and rats (Kabuto *et al.*, 2004; Gamez *et al.*, 2014).

3.2.2. The Protective Effect of SO against the BPA Effect on Relative Liver Weight

The relative weight of liver of the male mice treated with BPA and SO are summarized in Table 2. Results showed that the oral administration of SO according to the three regimens of treatment caused a significant increase ($P \leq 0.05$) in the relative weight of liver compared with the BPA-treated male mice. These results are in agreement with Hussien *et al.* (2013) who reported that the administration of SO with cypermethrin resulted in significant protection against its toxicity on body and organ weights of rats.

Table 2. Means and standard deviations of relative liver weight of male mice treated with BPA and/or SO for 28 consecutive days.

Treatments	Relative liver weight(g/100g)
Control	3.38±0.05^{ab}
Vehicle	3.39±0.05^{a}
SO	3.41±0.04^{a}
BPA	3.19±0.05^{d}
SO before BPA	3.34±0.06^{abc}
SO with BPA	3.28±0.10^{c}
SO after BPA	3.30±0.05^{bc}

Data are expressed as means ± SD. Mean values in the same column within each parameter bearing the same superscript do not differ significantly ($P \leq 0.05$).

3.3. Genetic Investigation

3.3.1. BPA's DNA Damage

As shown in Table 3, the mean values of tailed cells, tail length, tail DNA percentage and olive tail moment in liver cells of male mice treated with BPA were significantly increased than the control. These results came to a consensus with Tiwari *et al.* (2012) who found a significant increase in the micronuclei occurrence, structural chromosomal aberrations in the cells of bone marrow and DNA damage in the lymphocytes of adult rats treated with BPA in various doses (10 µg, 5 mg and 50 mg/kg b wt). In addition, a significant increase in the levels of chromosome aberrations and DNA damage in Chinese hamster ovary (CHO) cells exposed to BPA was observed by Xin *et al.* (2015). The alkaline comet assay is utilized in different research areas as a quantitative and qualitative technique to measure the DNA strand breaks in the cells (Gedik et al., 1998). The mechanism of BPA genotoxicity might work through the oxidative stress induction and the depletion of antioxidant enzymes (Meeker *et al.*, 2010). In addition, the micronuclei induction might be attributed to the BPA aneugenic effect (Hunt *et al.*, 2003; Quick *et al.*, 2008).

3.3.2. The Protective Effects of SO against BPA's DNA Damage

Significant alleviation ($P \leq 0.05$) in the mean values of tailed cells, tail length, DNA percentage in tail and olive tail moment as a result of the SO administration through the three regimens of treatment was observed (Table 3). In addition, data clearly illustrated that the treatment of mice with SO before BPA was the best regimen in restoring the observed DNA alterations (in liver tissue) followed by the administration of SO after the BPA treatment whereas, the co-administration of both SO and BPA was the least effective.

Several studies are in harmony with these results, Chattopadhyay *et al.* (2010) who revealed that the co-administration of sesame lignans and nicotine tartrate for fifteen days minimized the percentage of nicotine DNA damage in rat liver. Arumugam and Ramesh (2011) demonstrated that pretreatment with SO showed reduction in DNA damage induced by 4-Nitroquinoline-1-oxide. In addition, Hussien *et al.* (2013) found that the treatment of female rats with SO and cypermethrin together for thirty consecutive days obviously alleviated the incidence of DNA damage in brain tissues**.**

The SO protective effects could be attributed to its lignans compounds namely sesamol, sesamin and sesaminol (Suja *et al.*, 2004), which are responsible for many of the physiological and biochemical properties as antimutagenic and antioxidant (Rong *et al.*, 2005). In addition, these results might be attributed to the antioxidant nature of phenolic compounds in the SO, which have antimutagenic, anticarcinogenic, and anti-inflammatory properties that might potentially be helpful in caring for the genome stability (Xie *et al.*, 2013).

Table 3. Comet assay parameters in liver cells of treated male mice with BPA and/or SO for 28 consecutive days

Treatments	Tailed cells (%)	Tail length (µm)	Tail DNA (%)	Olive tail moment (µm)
Control	12.00±1.00^{e}	7.66±0.80^{e}	13.53±0.51^{d}	1.03±0.08^{d}
Vehicle	12.73±0.54^{de}	8.56±0.41^{e}	13.15±0.22^{d}	1.12±0.04^{d}
SO	12.67±0.57^{de}	8.40±0.45^{e}	13.50±0.26^{d}	1.13±0.05^{d}
BPA	29.43±0.51^{a}	17.30±1.08^{a}	19.93±0.58^{a}	3.63±0.52^{a}
SO before BPA	14.00±1.00^{d}	11.26±0.30^{d}	15.03±0.25^{c}	1.70±0.06^{c}
SO with BPA	18.67±0.55^{b}	13.93±0.31^{b}	16.76±1.50^{b}	2.20±0.14^{b}
SO after BPA	17.00±1.00^{c}	12.90±0.10^{c}	15.16±0.47^{c}	1.95±0.05^{bc}

Data are expressed as means ± SD. Mean values in the same column within each parameter bearing the same superscript do not differ significantly ($P \leq 0.05$).

3.4. Histopathological Examination

3.4.1. BPA's Histopathological Alterations

Oral administration of BPA for twenty-eight consecutive days caused several histopathological alterations in the liver of male mice. The examination of the mice liver sections treated with BPA revealed marked congestion of the hepatic blood vessels as well as marked vacuolar degeneration of the hepatocytes with many necrotic cells (Figure 1 B and C). The investigation of histopathological alterations in the liver treated with BPA, established the genetic findings as well. These data are confirmed by Korkmaz *et al.* (2010) who noticed hepatic necrosis and congestion in male rats submitted to BPA treatment (25 mg/kg/day) for 50 day. Moreover, Ahmed *et al.* (2015) found that the treatment with BPA of adult male rats at the dose of 150 mg/kg for seventy consecutive days caused hepatic histopathological alterations.

These histopathological alterations may be related to the induction of DNA damage by BPA in these tissues. Moreover, it has been reported that BPA can cause oxidative damage in rat organs by generating ROS (Chitra *et al.*, 2002; Kabuto *et al.*, 2004). In addition, Sangai and Verma (2012) found that BPA caused changes in the activities of ATPase in the liver and kidney of mice thereby causing a reduction in the ATP production which causes necrosis.

3.4.2. The Protective Effects of SO against BPA's Histopathological Alterations

The microscopical examination of liver tissues revealed that SO through the three regimens of treatment with BPA

effectively improved the histological alterations induced by BPA especially the regimen of administration of SO before the BPA treatment. Liver histopathological examination of the SO-treated mice for twenty-eight successive days before the BPA treatment showed a mild degree of hepatocellular degeneration and necrosis (Figure 1 D). While, liver sections of the mice co-treated with SO and BPA showed centrilobular necrosis and degeneration of the hepatic cells (Figure 1 E). The examination of the liver sections of SO-treated mice for twenty-eight successive days after the BPA treatment showed congestion of the central veins and blood vessel moderate necrobiotic in the hepatocytes (Figure 1 F).

These findings are in agreement with those of Periasamy *et al.* (2012) who confirmed the protective effects of SO against monocrotaline-induced liver histopathological changes in rats. In addition, Soliman *et al.* (2015) noticed that the treatment of rats with SO ameliorated cypermethrin induced degenerative changes in the liver tissue. The protective role of SO may be attributed to the antioxidant nature of vitamin E that can prevent cell oxidative damage by preventing the oxidation of unsaturated fatty acids (Kim *et al*., 2012). In addition, SO contains active phenolic lignans such as sesamin, sesamol, and sesamolin, which are known as hepato-protective components (Periasamy *et al.*, 2014).

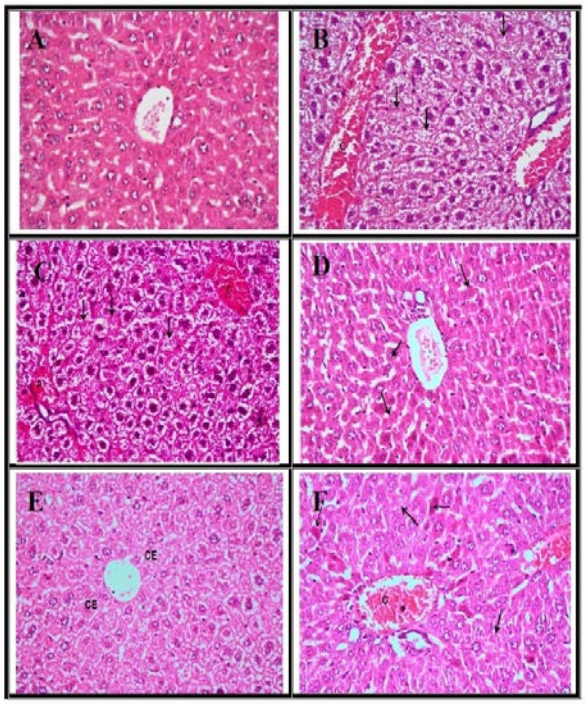

Figure 1. (A) Liver of control mice appearing within normal limits. B & C) Liver of BPA- treated mice showing hepatic blood vessels congestion (C), marked vacuolar degeneration of the hepatocytes with many necrotic cells (arrow), congestion of the central vein (C) and portal blood vessels (P) with marked hepatocellular vacuolar degeneration and necrosis (arrow). D) Liver of SO-treated mice before BPA showing mild degree of hepatocellular degeneration and necrosis (arrow). E) Liver of SO-treated mice concurrently with BPA showing centrilobular (CE) necrosis and degeneration of the hepatic cells. F) Liver of SO-treated mice after BPA treatment showing congestion (C) of the portal blood vessels and hepatocytes moderate necrobiotic changes (thick arrow) in the hepatocytes, Notice apoptotic cells (thin arrow) (H&E X400).

3.5. Biochemical Analysis

Finally, to confirm the protective effects of SO against the histopathological alterations induced by BPA in the liver tissue, serum AST and ALT were determined.

3.5.1. The Effects of Bisphenol A on Liver Enzymes

Results in Table 4 showed that the oral administration of BPA caused a significant increase ($P \leq 0.05$) in serum AST (49.33±1.52) and ALT (42.33±1.52) levels compared to control (29.67±1.15 and 24.67±1.15, respectively). These findings are in conformity with Korkmaz *et al.* (2010) who revealed that aspartate transaminase (AST) and alanine transaminase (ALT) levels were increased in male rats exposed to BPA (25 mg/kg) for fifty days. The high level of AST and ALT was accompanied by free radical generation and alteration in the liver tissue (Gaskill *et al.*, 2005).

3.5.2. Sesame Oil Protective Effects against BPA's Liver Dysfunction

Compared to the BPA group, the oral administration of SO through the three regimens of treatment with BPA significantly attenuated the liver enzymes (Table 4). Furthermore, the treatment of mice with SO at regimen before the BPA administration resulted in the best alleviation of the serums ALT and AST levels followed by the administration of oil at regimen after and during the BPA treatment. These findings are in accordance with Periasamy *et al.* (2012) who reported that SO significantly alleviated the AST and ALT levels in monocrotaline-treated rats.

Table 4. Biochemical measurements in the serum of BPA and/or SO treated male mice for 28 consecutive days.

Treatments	AST(U/L)	ALT(U/L)
Control	29.67±1.15 ^{de}	24.67±1.15^{c}
Vehicle	31.00±2.00^{cde}	23.67±0.57^{c}
SO	27.33±0.57^{e}	24.67±1.15^{c}
BPA	49.33±1.52^{a}	42.33±1.52^{a}
SO before BPA	32.00±1.73^{cd}	27.67±3.51^{c}
SO with BPA	41.00±2.64^{b}	34.67±2.08^{b}
SO after BPA	34.33±4.50^{c}	27.68±5.03^{c}

Data are expressed as means ± SD. Mean values in the same column within each parameter bearing the same superscript do not differ significantly ($P \leq 0.05$).

4. Conclusion

It can be concluded that BPA has the potential to produce genotoxic effects, and histopathological alterations in the liver tissue of male mice in addition to biochemical alterations. Accordingly, strict limitations on the use of this compound must be put especially in food contact materials. Sesame as a valuable seed oil appears to have abundant beneficial properties for applications in food. In addition, it can be considered as an excellent novel and multi-purpose ingredient in several industrial, cosmetic, and pharmaceutical products. Data reported herein illustrated that sesame seed oil can used as an antioxidant to attenuate the hepatotoxicity of BPA. Subsequently, it can be

categorized as edible oil with a high potential of antioxidant activity.

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JJBS

Volume 11, Number 4, September 2018
ISSN 1995-6673
Pages 469 - 473

Jordan Journal of Biological Sciences

Short Communication

A Comparative Analysis of the Suppressor Activity of *Tobacco mosaic virus* Proteins in the Tomato Plant

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Received December 18, 2017; Revised January 17, 2018; Accepted January 20, 2018

Abstract

Plant viral suppressors are proteins which have the capability of inhibiting induced host antiviral RNA interference. RNA silencing is part of the cellular machinery that controls the translation of transcripts, and plays a major role in the defense against virus infections. *Tobacco mosaic virus* (TMV)-encoded proteins (Replicase, Movement and Coat proteins) were expressed in pCAMBIA1302 vector, and the suppressor strength was checked on the tomato plant. The suppression activity was detected by assaying for GFP fluorescence at 3dpi, 5dpi, 7dpi and 10dpi. Phenotypic observations were corroborated with semi-quantitative RT-PCR. The results indicated that TMV suppressors of the gene silencing are the coat protein.

Keywords: Tomato; TMV; Co-agroinfiltration; RNA Silencing Suppressor, GFP Silencing Assay

1. Introduction

Most eukaryotic organisms are equipped with an RNA silencing system that is being the most important and significant antiviral defense mechanisms (Hamilton *et al.*, 2002; Matzke and Birchler, 2005).

For plant-viral infections, viruses need to replicate their own genomes inside the cell. However, once the virus infects the plant, the host posttranscriptional gene silencing (PTGS) machinery is activated as a first line of plant defense response (Marathe *et al.*, 2000; Waterhouse *et al*., 2001). RNA silencing suppressors (RSS) are proteins encoded by many plant viruses having the ability to suppress the silencing based virus defense strategy and play an important role in virulence (Brigneti *et al.*, 1998; Baulcombe, 2002; Das and Sanan-Mishra, 2014). The RSS are interacting with the RNA silencing machinery components resulting in a breakdown of the host antiviral defense response (Das and Sanan-Mishra, 2014). However, inactivation of the RSS activity will lead to the recovery of plants from the viral infection (Silhavy *et al.*, 2002; Ziebell *et al.*, 2007).

Various RSS have been reported from diverse viruses and a lot of work is being done to understand their mechanism of action. Among the tobamoviruses, the RSS were shown to bind double-stranded small RNAs, inhibit 2′-O-methylation of small RNAs, and prevent the formation of RNA-induced silencing complexes (Akbergenov *et al.*, 2006; Vogler *et al.*, 2007).

Tobacco mosaic virus (TMV; genus Tobamovirus; family Virgaviridae) is a positive-sense single strand RNA virus (Meshi *et al.*, 1992). The TMV genome encodes three proteins: the replicase components proteins (RP; 130 kDa and 180 kDa) required for transcription and replication, the movement protein (MP; 30 kDa) required for cell-to-cell virus movement, and the coat protein (CP; 17.5-kDa) required for virus coat formation (Dorokhov *et al.*, 1994). The sequence of 130 kDa includes the methyltransferase and RNA helicase motifs, while the sequence of 180 kDa includes the methyltransferase, RNA helicase and RNA-dependent RNA polymerase motif. So the 180 kDa sequence can read-through protein of the 130 kDa open reading frame. TMV infects tomato plants systemically causing heavy losses in the tomato yield worldwide (Sikora *et al.*, 1998).

In addition to its role in the virulence and activation of pathogen-related genes, it was demonstrated that CPs may play an important role in the suppression of the RNA silencing, viral replication, attachment to the site host, cell-to-cell movement, and symptom development (Callaway *et al.*, 2001; Culver, 2002).

For the RSS assays, there are two common methods widely used, the first one is called the Agro-infiltration method which depends on the reversal transient suppression assay and in which model plants stably silenced for a reporter gene like GFP are used. Restoration of the reporter gene expression indicates that the tested construct encodes an RSS. The second method is a modification method called co-Agroinfiltration, in which two *Agrobacterium* strains are used (Johansen and Carrington, 2001; Takeda *et al.*, 2002). This work is aimed at assessing and identifying the suppressor activity of the

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three TMV-encoded proteins in tomato plants using co-agroinfiltrating method.

2. Material and Methods

2.1. Primer Design and TMV Genes Amplification

Based on the published sequence of TMV genome (Ac# KF972436), two degenerate primers were designed by NCBI Primer blast software (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) to amplify the full length of replicase (RP; 130 kDa) and movement proteins. The primers sequence was checked by the NCBI-BLAST online software. The source of the TMV used in this study was isolated by our laboratory in Egypt and maintained continuously on *N. banthemiana*. One microgram of viral RNA was used to synthesize cDNA using oligo (dT) primer with Superscript reverse transcriptase (Invitrogen, USA) according to the manufacturer's instructions. A volume of 1 µl of cDNA was used for the PCR amplification of the replicase, movement and coat protein genes using TMV-specific primers (Table 1). The PCR amplified products were analyzed on 1.2 % agarose gel.

Table 1. Primer sequences and their relative position in the respective TMV genome

Gene		Primer sequence 5'……..3'	Position	Amplification Size (bp)	Reference
CP	Forward	ATTTAAGTGGASGGAAAAVCACT	5542 to 5562	695	Letscher *et al.*, 2002
	Reverse	CGGTCAGTGCCGAACAAGAA	6236 to 6213		
MV	Forward	GCTCGCAGATTTTGATTTTG	3872 to 3891	1827	This study
	Reverse	ACGAACTGAGATGGAGTAGT	5698 to 5679		
RP	Forward	CAATGATCTAGCAAAGCGTCG	89 to 109	3356	This study
	Reverse	CAGAAATATCACCAGTCTTTGGC	3444 to 3422		

2.2. Plasmid Construct

Each individual gene of the RP, MV and CP genes were purified, and cloned into a pGEMTeasy vector (Promega, USA) to adapt ligation site with the pCAMBIA1302-GFP binary vector sites. The three recombinant plasmids were transformed into *Agrobacterium tumefaciens* strain LBA4404 to start the agroinfiltration study after the ligation and cloning of the three modified genes into pCAMBIA1302 under the CaMV 35S promoter. The transformants were checked by plasmid isolation, restriction enzyme analysis and colony PCR.

2.3. Preparation of Agrobacterium Competent Cells and Transformation Protocol

Agrobacterium tumefaciens strain LBA4404 was grown in 5 mL YEM medium supplemented with 50 mg L^{-1} streptomycin and 25 mg L^{-1} rifampicin at 28 ^{o}C in a shaking incubator at 180 rpm. The primary culture was added to 50 mL YEM medium supplemented with streptomycin and rifampicin, and was incubated at 28 ^{o}C with shaking for 4-5h till an optical density (OD) reaching 0.5-1.0 at 600 nm. The culture was chilled on ice for thirty minutes, and the cells were harvested by centrifugation at 5,000 rpm, at 4 ^{o}C. The pellet was suspended gently in 20 mL cold, sterile 100 mM $CaCl_2$, and was kept on ice for thirty minutes. The *Agrobacterium* suspension was centrifuged at 5000 rpm, and the pellet was re-suspended in 1 mL of chilled, sterile $CaCl_2$ plus 1 ml of 50 % sterile glycerol, and was mixed gently by pipetting. Each 100 µL competent cells were aliquoted into a sterile microfuge tube, and were stored at -80 °C for further use.

For transformation, *Agrobacterium* competent cells were thawed by being kept on ice for ten minutes. A quantity of 1-2 µg plasmid DNA was added to the *Agrobacterium* cell tube, and was mixed well and incubated on ice for thirty minutes. The cells were quickly frozen by immersing the microfuge tube in liquid nitrogen for one minute; heat shocked at 37 ^{o}C in the water bath for five minutes, and were immediately kept on ice again for five minutes. A volume of 1 mL LB medium was added to the cells, and was incubated at 28 ^{o}C for three-five hours with shaking. A 100µl of the suspension was spread onto LB-agar plate containing 50 mg L^{-1} kanamycin, 50 mg L^{-1} streptomycin and 25 mg L^{-1} rifampicin, and was incubated at 28 ^{o}C. Transformed colonies that appeared after two-three days were screened by colony PCR, plasmid isolation, and restriction enzyme digestion analysis.

2.4. Co-agroinfiltration and GFP Detection

Seeds of Heinz 1706 tomato (*Solanum lycopersicum* L.) cultivar were grown in a greenhouse under controlled conditions. At forty-five days, *A. tumefaciens* harboring the appropriate plasmid construct was infiltrated according to Karjee *et al.*, (2008) method with some modification. Briefly, *A. tumefaciens* was inoculated to LB medium with appropriate antibiotics, and was grown until OD 600 reached one. Cells were harvested by centrifugation at 4 ^{o}C with 5000 rpm and were re-suspended in YEM to adjust a final OD600 ≈ 0.8-1.2. With the help of a finger on the dorsal side of the tomato leaf, pressure infiltration (by a syringe without a needle on the leaf ventral side) was carefully applied. At 3, 5, 7, and 10 days, post-inoculation (dpi) the infiltrated leaves were collected and GFP fluorescent was visualized under fluorescent UV.

2.5. GFP Reporter Transcript

For semi-quantitative RT-PCR, 1 µg of total RNA isolated from the infiltrated leaf tissues at different

intervals was used to synthesize cDNA. After the treatment of cDNA with DNaseI for thirty minutes, 1 µL was used as a template for PCR amplification. The PCR program with initial denaturation at 95°C for three minutes followed by thirty-five cycles at 94˚C for thirty seconds, 58˚C for thirty seconds, 72˚C for thirty seconds, and final extension at 72˚C for five minutes. The tomato actin gene forward 5'-ATGCCATTCTCCGTCTTGACTTG-3', reverse 5'-GAGTTGTATGTAGTCTCGTGGATT-3' primer (Naqvi *et al.,* 2010) was applied as a reference gene to evaluate the cDNA content. RT-PCR products were analyzed by electrophoresis in 1.2 % agarose gel, and the band intensities were quantified using Alpha Imager Imaging System.

3. Results and Discussion

To identify the potential RNA silencing suppressors (RSS) of TMV, the three open reading frames of RP, MV and CP were amplified using RT-PCR with specific primers. An individual amplicon with a molecular size of about 695 bp for CP, 1827 bp for MV, and 3356 bp for RP genes (Figure 1) was cloned into the pCAMBIA1302 binary vector under CaMV 35S promoter, and was then transformed into in the *A. tumefaciens* strain LBA4404.

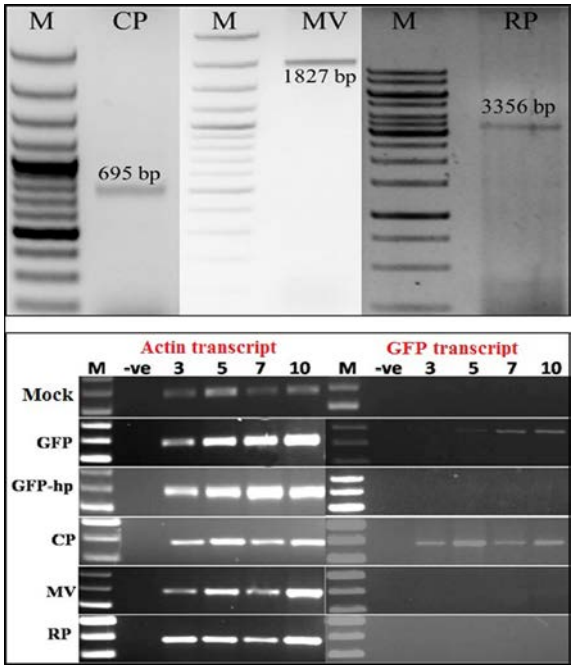

Figure 1. Agarose gel electrophoresis (1.2%) in TAE buffer stained with ethidium bromide showing (Upper): RT-PCR amplification of TMV-coat protein (CP), TMV-movement protein (MV), and TMV-replicase protein (RP) of TMV-infected tomato; (Down): analysis of GFP transcripts in total RNA obtained from tomato leaves infiltrated six treatments that referred by mock (*Agrobacterium* empty), GFP (pCAMBIA1302), GFP-hp (pCAMBIA1302 plus GFP-hairpin), CP (GFP-hp+CP), MV (GFP-hp+MV) and RP (GFP-hp+RP).

Based on the transient assay for the suppression of GFP silencing (Johansen and Carrington, 2001), the RSS activity of each gene was estimated. The construct of pCAMBIA1302 carrying the green fluorescent protein was used as GFP expressing positive control. Mixture with an equal volume of the two constructs of pCAMBIA1302 and GFP-hairpin (GFP-hp) co-infiltrated together were used as GFP silencing control. For RP, MV and CP, GFP-hp construct was mixed and co-agroinfiltrated with pCAMBIA1302-RP, pCAMBIA1302-MV and pCAMBIA1302-CP. The infiltrated zones were analyzed for the suppression of GFP silencing after 3, 5, 7 and 10 dpi (Figure 2). The results of this study revealed that the leaves infiltrated by pCAMBIA1302 alone exhibited a GFP fluorescence in the infiltrated zones at 5dpi, and the fluorescence increased by 7dpi and 10dpi (Figure 2). These initial observations indicated that the transient infiltration of pCAMBIA1302 in tomato leaves resulted in GFP expression, and this increased with time till 10dpi. This result is in agreement with earlier observations by Kokkirala *et al.,* (2010). However, no color for the GFP expression was detected in leaves infiltrated with mock (*Agrobacterium* free), or co-infiltrated with GFP-hp at all time (Figure 2). The GFP-hp results in the production of siRNA against GFP and these silence its expression through the PTGS (Simmer *et al.,* 2010). The regions co-infiltrated with GPF-hp+CP showed enhanced expression starting at 3dpi, and increased with time reaching a maximum at 10dpi (Figure 2). This showed that CP could suppress the hp-induced silencing resulting in greater accumulation of GFP. In addition, the GPF-hp+RP and GPF-hp+MV did not show any green color for the GFP expression (Figure 2). This data suggested that the RP and MV proteins lacked the RSS activity, or it was too weak to be visually detected.

By comparing the three proteins together, it was observed that the CP only had RSS activity, so it can be designated as the stronger suppressor protein of the TMV genome. In addition, the CP itself in the absence of other TMV-encoded proteins was able to suppress host silencing machine. These results are similar with the previous data that reported the CPs of several plant viruses that have been identified as suppressors of gene silencing (Roth *et al.,* 2004; Soosaar *et al.,* 2005; Ren *et al.,* 2005). These results do not agree with Conti *et al.,* (2017) who demonstrated that the replicase protein carried the TMV suppression of silencing activity. Although those authors noted that during the TMV infections, RNA decay pathways were induced by the action of movement and coat protein.

Semi-quantitative RT-PCR was applied to quantify the degree of suppressor activity based on the transcriptional of the GFP reporter. Molecular analysis has confirmed the results of GFP silencing assay. It was observed that there was no GFP transcript accumulation detected in the infiltrated regions with mock, GFP-hp and TMV-RP (Figure 1). On the other hand, GFP transcript was detected in both GFP and GFP-hp-CP infiltrated leaves (Figure 1). Qu *et al*., (2003); Thomas *et al*., (2003) and Meng *et al*., (2006) reported that the CP of both *Turnip crinkle virus* and *Chlorotic ringspot virus* are the strong suppressors of RNA silencing.

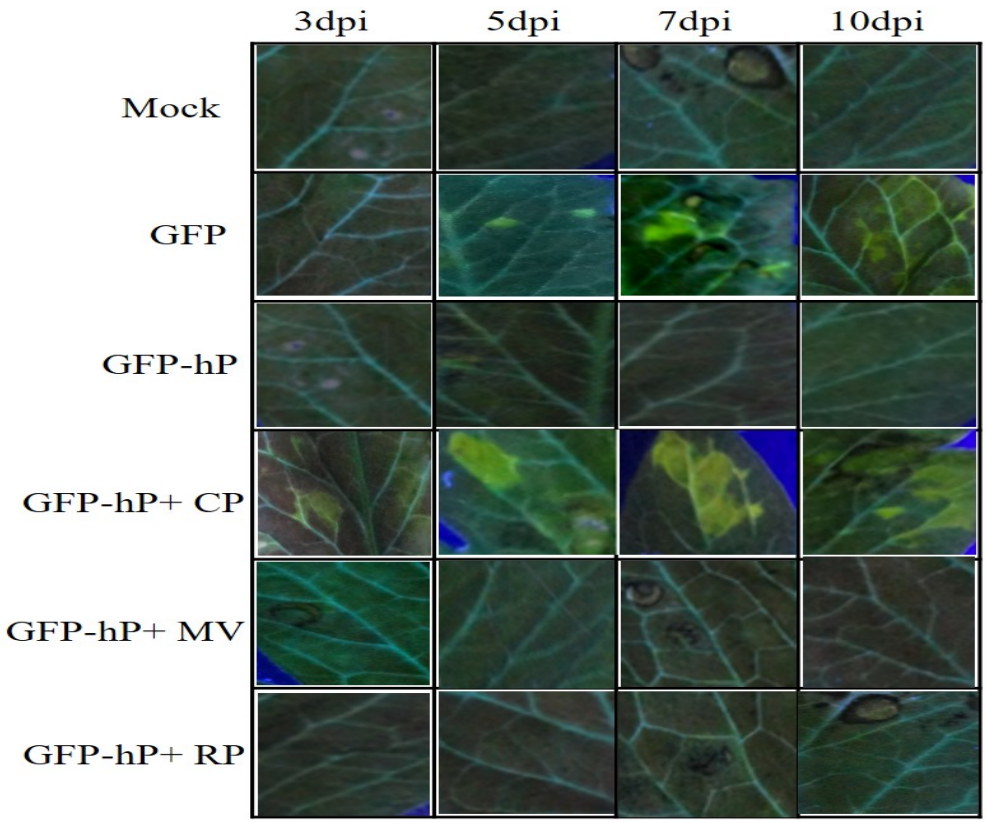

Figure 2. GFP fluorescent in tomato leaves under UV. Tomato leaves were co-agroinfiltrated with six treatments that referred by mock (*Agrobacterium* empty), GFP (pCAMBIA1302), GFP-hp (pCAMBIA1302 plus GFP-hairpin), GFP-hp+CP, GFP-hp+MV and GFP-hp+RP.

At 3dpi, the GFP transcript band was detected only with GFP-hp-CP infiltration, which indicated the suppression activity of CP that starts early, and could suppress the hp-induced silencing of the GFP construct (Figure 1). The band intensity was highly increased after 5dpi and 10dpi (Figure 1). While the GFP transcript with GFP construct was detected as a faint band at 5dpi, but it increased at 10dpi (Figure 1). This may be due to the initiation of host-induced RNAi response against the infiltrated GFP constructs. The results are in harmony with those results obtained by Das and Sanan-Mishra, (2014) and not compatible with the results obtained by Csorba *et al.*, (2007). At 7 dpi till 10 dpi, the GFP transcription with MV protein was detected as very low bands (Figure 1). Luna *et al.*, (2017) reported that the *Beet curly top virus* V2 protein is involved in the virulence determination and viral spread acted as a potent PTGS suppressor.

From the obtained results we can concluded that the 17.5 kDa coat protein of TMV is a potent silencing-suppressor protein, such results will give attention to detect a suppressor-host virus relationship, and can help to establish a strategy for gene expression aiming to enhance the plant viral immune system.

Acknowledgements

Abdelkhalek A. would like to thank Arturo Falaschi International Centre for Genetic Engineering and Biotechnology for granting the post-doctoral Fellowship. The authors thank ICGEB (New Delhi, India) for providing all the necessary facilities to perform the research work.

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