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EDITORIAL PREFACE

Jordan Journal of Biological Sciences (JJBS) is a refereed, quarterly international journal financed by the Scientific Research and Innovation Support Fund, Ministry of Higher Education and Scientific Research in cooperation with the Hashemite University, Jordan. JJBS celebrated its 12th commencement this past January, 2020. JJBS was founded in 2008 to create a peer-reviewed journal that publishes high-quality research articles, reviews and short communications on novel and innovative aspects of a wide variety of biological sciences such as cell biology, developmental biology, structural biology, microbiology, entomology, molecular biology, biochemistry, medical biotechnology, biodiversity, ecology, marine biology, plant and animal biology, plant and animal physiology, genomics and bioinformatics.

We have watched the growth and success of JJBS over the years. JJBS has published 11 volumes, 45 issues and 479 articles. JJBS has been indexed by SCOPUS, CABI's Full-Text Repository, EBSCO, Clarivate Analytics- Zoological Record and recently has been included in the UGC India approved journals. JJBS Cite Score has improved from 0.18 in 2015 to 0.58 in 2019 (Last updated on 16 March, 2020) and with Scimago Institution Ranking (SJR) 0.21 (Q3) in 2018.

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

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

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A Molecular Docking Study of *Aloysia citrodora* Palau. Leaf Essential Oil Constituents towards Human Acetylcholinesterase: Implications for Alzheimer's disease

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Abstract

Aloysia citrodora essential oil has been described as a potential new candidate for treating Alzheimer's disease (AD). It displays a range of pharmacological properties, including acetylcholinesterase (AChE) inhibition worthy of investigation. The essential oil of the fresh leaves of *A. citrodora* was obtained by hydrodistillation, and the composition of the essential oil was investigated using GC-MS (Gas Chromatography-Mass Spectrometry). Sixty compounds were identified, which were composed of oxygenated monoterpenes and sesquiterpenes. In the evaluation of the anticholinesterase activity of the essential oil through virtual screening, molecular docking and bioassay were used. Seven main components were identified namely caryophyllene oxide, geranyl acetate, β-sesquiphellandrene β-curcumene, γ-/ar-curcumene, β-/(Z)-α-bisabolene, *trans*-calamenene, and β-sesquiphellandrene. Three compounds (geranyl acetate, caryophyllene oxide and bisabolene) were available commercially and assessed for AChE inhibitory activity to validate the approach. The dose-response relationship showed that AChE inhibitory with IC_{50}= 5.3 µM for caryophyllene oxide and 244.5 µM for geranyl acetate. The molecular docking study revealed that *A. citrodora* may yield new selective inhibitors of AChE from plant-originated essential oils.

Keywords: *Aloysia citrodora*, Essential oil, Molecular docking, Acetylcholinesterase inhibition, Alzheimer's disease.

1. Introduction

Alzheimer's disease (AD) is known as the most prevalent age-associated neurodegenerative disorder and the major cause of dementia worldwide. The estimated number of individuals with dementia is 46.8 million and it is expected to reach 74.7 million by 2030 according to the 2015 World Alzheimer Report (Zhu *et al.*, 2018). It is a progressive disease characterized by a loss of basal forebrain neurons and reduced cortical and hippocampal level of acetylcholine (ACh).

AChE, is a key modulator for cholinergic neurotransmission, where it terminates impulse transmission at cholinergic synapses, by hydrolyzing the neurotransmitter acetylcholine. AD is accompanied by a decline in cholinergic functioning and, thus, to raise the level of ACh, a key enzyme in the breakdown of the ACh is a way of compensation for the lowered concentrations of ACh (Anand *et al*., 2017). The relation between the observed cholinergic dysfunction and AD severity provides a rationale for the therapeutic use of acetylcholinesterase inhibitors (AChEIs).

Up till now, no effective pharmacotherapeutic options for disease prevention exist. Only limited symptomatic treatment of AD (Habash *et al*., 2017), is available, with three AChE inhibitors, *e.g*. rivastigmine, donepezil, and galantamine, in clinical use (Jiang *et al.*, 2017). Plant secondary metabolites have been the center of attention in the search for new bioactive leads due to their chemical diversity and wide range of therapeutic effects (Korábečný et al., 2018). An increasing amount of evidence in the literature shows that essential oils (EOs) are a good source of several bioactive compounds targeting AD. They are composed of enormously complex chemical composition with volatile-derived compounds, including mostly monoterpenes and sesquiterpenes (Abuhamdah *et al*., 2015).

Lemon verbena - *Aloysia citrodora* Palau (Verbenaceae) is an aromatic plant native to Argentina, Paraguay, Brazil and various parts of the Middle East (Bahramsoltani *et al.*, 2018). The plant extracts are valuable for medicinal preparations. The plant has a gentle sedative action, a mild tonic effect upon the nervous system, and helps to counter anxiety and depression (Gil *et al.*, 2007). *A. citrodora* L. EO has been previously shown

* Corresponding author e-mail: smaabuhamdah@gmail.com.

* List of abbreviations : AD: Alzheimer's disease, ACh: Acetylcholine, AChE: Acetylcholinesterase, hAChE: human acetyl cholinesterase, ChEIs: Cholinesterase inhibitors, GC-MS: Gas chromatography–mass spectrometry, FID: Flame ionization detector, IC50: is the concentration of an inhibitor where the response (or binding) is reduced by half.

to possess a range of useful neuropharmacological properties, including potent AChE inhibitory activity and *in vitro* neuroprotective potential (Abuhamdah *et al*., 2015).

Molecular docking and binding prediction of two molecules in 3D space is a newly established model system available in the discovery of novel chemical binding hits to a specific protein target. Adopting this approach, the study of the interaction between a chemical and a protein of interest at the atomic level, permits the characterization of the binding site location in target proteins. Identifying the "best-fit" orientation of a ligand and a particular protein, by minimizing the free energy of the system, can predict both the affinity and efficacy of the molecule.

The aim of the present study is to identify the chemical composition of *A. citrodora* leaf essential oil, evaluation of the AChE inhibitory activity and molecular docking of main components of the oil inside human AChE in order to find novel inhibitors towards AD.

2. Material and Methods

2.1. Chemicals

All chemicals, reagents and solvents were purchased from Sigma-Aldrich Chemical Co. (USA). AChE from electric eel, Type V-S, Ellman's reagent, acetylthiocholine iodide, eserine, caryophyllene oxide, geranyl acetate, and bisabolene were purchased from Santa Cruz Biotechnology (Dallas, USA).

2.2. Collection of plant material, essential oil extraction and GC-MS Analysis

The leaf parts of *A. citrodora* were collected from Jordan in April, 2015. The botanical identity of the plant specimen was confirmed by Prof. of Pharmacognosy and Phytochemistry, Suleiman Olimat at Department of Pharmaceutical Sciences, Faculty of Pharmacy, Jordan University of Science & Technology, Jordan. It was authenticated to be *Aloysia citrodora* Palau belonging to family Verbenaceae. A voucher specimen of the plant is stored in Department of Pharmaceutical Sciences, The University of Jordan. 500 g of the leaves was subjected to hydrodistillation for 6 h, according to the standard procedure described in the European Pharmacopoeia and stored at 4 °C in the dark until further study. Analytical gas chromatography was carried out on a Perkin-Elmer USA, equipped with a flame ionization detector (FID) and mass spectrometry GC-MS analyses. Chemical constituents were identified by comparison of their mass spectra retention indices with those of the literature data as previously described (Abuhamdah *et al*., 2015).

2.3. Molecular Docking

The co-crystal structure of human AChE with donepezil (PDB code: 4EY7) was retrieved from Protein Data Bank. Molecular docking of compounds into the active site of hAChE was performed through Genetic Optimization for Ligand Docking (GOLD v3.0.1) software (CCDC, Cambridge, U.K), which helps to predict the ligand conformational flexibility by genetic algorithm. Empirical scoring function was optimized using ChemPLP for pose prediction and calculations of the binding affinity between two molecules, after they have been docked.

2.4. Enzymatic Assay for AChE Inhibition

AChE inhibitory activities of the test compounds was determined by the Ellman's colorimetric method (Ellman *et al.,* 1961). Briefly, reactions involved adding 140 µl Tris buffer, 20 µl DTNB, 20 µl test solution, and 20 µl AChE solution in a 96-well microplate, and incubation for 15 min at 25 °C then 10 µl ACh added and the formation of the yellow color was monitored at 412 nm utilizing a 96-well microplate reader (BioTek ELx800). The percent of inhibition was determined by using the formula: $(1\text{-}A_I/A_C) \times 100$, where A_I and A_C are the respective enzyme inhibitory activity and control. The experiments were carried out in triplicate. Eserine (physostigmine) was used as the reference drug.

2.5. Statistical analysis

IC_{50} values were determined graphically from inhibition curves using the GraphPad Prism Software version 4.00 (GraphPad Software, Inc., San Diego, CA, USA) with the log (inhibitor) *vs.* response (variable slope) function.

3. Results

3.1. Phytochemical profile of A. citrodora essential oil

We have already reported the chemical profile of the EO from *A. citrodora* (Abuhamdah *et al*., 2015). In total, 63 compounds were identified representing 93% of the total oil. The oil was characterized by a high content of monoterpenes and moderate levels of sesquiterpenes; the main compounds included geranyl acetate, curcumene, spathulenol, caryophyllene oxide, limonene, geranial, neral, and 1,8-cineole, where this chemical profile was the basis of the *in silico* study in hand (Abuhamdah *et al*., 2015).

3.2. Structure-based virtual screening of A. citrodora EO constituents for AChE inhibition

To perform the study described herein, the crystallographic structure of human AChE (hAChE) (PDB: 4EY7) was downloaded from protein data bank (Figure 1). The structure of hAChE in complex with donepezil is depicted as a ribbon diagram (Cheung *et al.,* 2012) (Figure 2). A centre was established around the determined binding site. 15Å radius was chosen to form the search space. Default settings for small molecule-protein docking were used throughout the simulations. Molecular docking GOLD version 3.0.1 was employed to investigate the binding mode, and the default parameters of genetic algorithms (GA) were applied to search for a reasonable binding conformation of the flexible ligands. The maximum number of GA runs was set to 10 for each compound. ChemPLP scoring function was used to evaluate the docking conformations. Docked conformations were saved in MOL2 format, and imported into Hermes for further analysis. The donepezil pose derived from GOLD was adopted as the reference compound for alignment. In order to consider the influence of crystallized water molecules on docking, donepezil was docked into the binding pocket with and without water molecules, separately docking conformations of donepezil superimposed with the X-ray crystal structure very well, which reflected that the co crystallized water had little impact on docking (Abuhamdah, 2014).

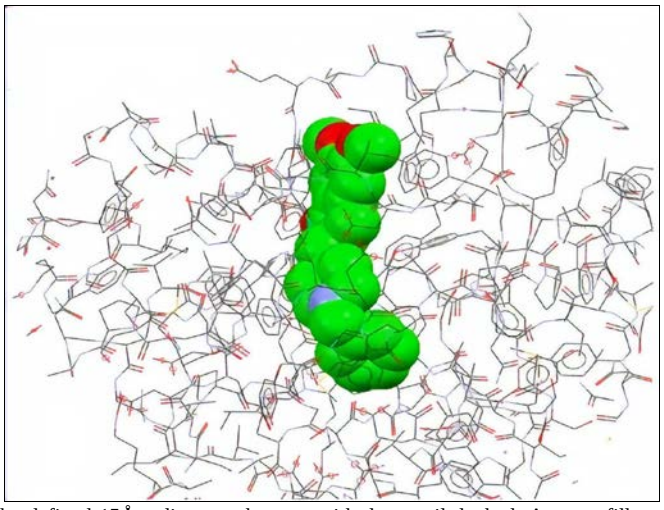

Figure 1. A magnification of the defined 15Å radius search space with donepezil docked: A space fill model. GOLD Molecular docking version 3.0.1.

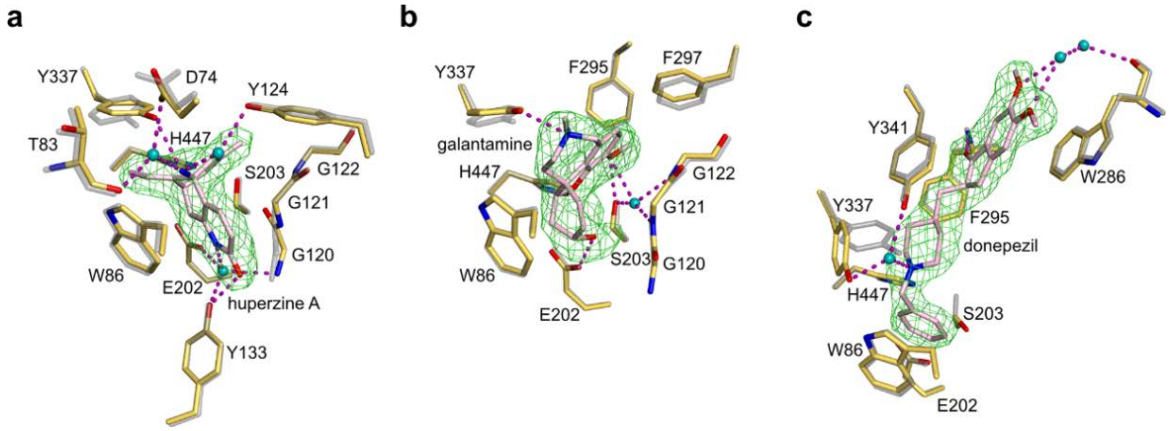

Figure 2. Close up views of the AChE active site with bound AChEI standards a) huperzine A, b) galantamine and c) donepezil. Carbons are in pink in the ligands and light yellow in residues of the complex [10]

Eighty-eight AChE inhibitors in this study (including donepezil, galantamine, huperzine, rivastigmine and tacrine) were taken from Cheung *et al.* (2012) all of which were reported by the same laboratory with similar experimental conditions and procedures to obtain bioactivity data for the compounds. The 3D chemical structures of these acetylcholinesterase inhibitors were sketched using ChemBioDraw Ultra (Ver12.0.2.1076 Cambridge Soft Corp., Cambridge, MA) then saved in mol file formats; these were considered as the training set. All chemical constituents detected in *A. citrodora* EO (Abuhamdah et al., 2015) were sketched using ChemBioDraw Ultra and also saved in mol file format. Each compound was tested for ten genetic algorithm runs, generating ten docking poses for each compound, with the top five conformations saved for analysis. Each docking run was repeated twice. The optimal docking protocol and scoring function was chosen, and the output options created sub-directories for each ligand conformation docked in order of best fit. The scores of these top hits had values comparable to those possessed by optimal conformations of huperzine A, rivastigmine, donepezil, and galantamine (the well-known alkaloid type of drug as AChE inhibitor) in the active site (Figure 2). The top scoring hits from *A. citrodora* EO constituents were imported to Hermes software to view crystal structures with docked ligands (Figures 3A, B, C, D, E, F and G). The top seven scoring hits showed high similarities in the protein residues with which they interact, both with themselves and known AChE inhibitors, such as huperzine A and donepezil, as shown in Table 1. The most common interactions were with TYR 341 + 337, PHE 338 and HIS 447, respectively. The majority of these interactions involved hydrogen bonding, hydrophobic and π-π interactions (Abuhamdah, 2014).

Seven hit compounds exhibit a good docking score when compared to the standard donepezil. *Trans*-calamenene has six stacking interaction with TYR337, TYR341, TRP286, HIS447 and ASP74. On the other hand, (Z)-α-bisabolene has five stacking interactions with TYR337, TYR341, TRP286, PHE 338 and VAL 294. Geranyl acetate and caryophyllene oxide both have four stacking interactions; these were TYR 341 PHE 338 PHE 295 HIS 447 and TYR337, TYR341, TYR124 and HIS447 respectively. β-curcumene, ar-curcumene, and β-sesquiphellandrene were active hits with three stacking interactions, with all sharing the interaction with TYR341. (β-curcumene TYR337, TYR341, HIS447; ar-curcumene TYR337, TYR341, VAL 294; β-sesquiphellandrene TYR341, PHE 338, VAL 294).

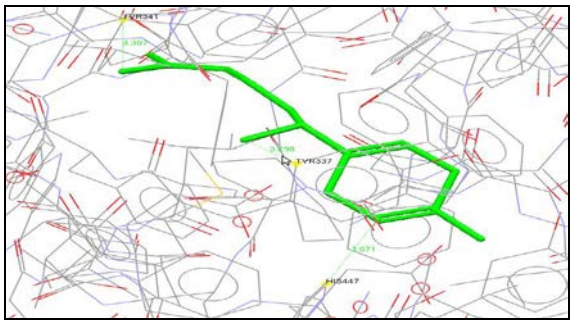

Figure 3A. 1st Ranked Hit: Close up views of the hAChE active site with bound **β-curcumene**, (carbon atoms in green) showing Ligand-Protein interactions points (yellow) at TYR 341, TYR337, HIS 447. β-curcumene had the highest hit score of PLP 75.64.

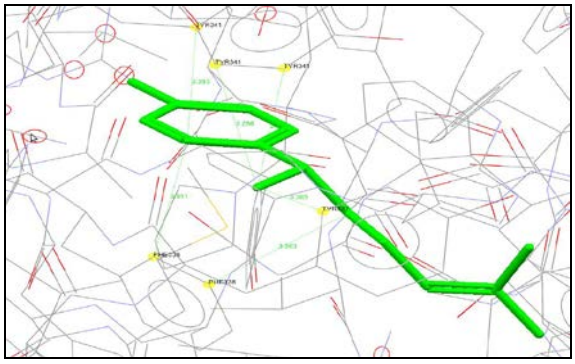

Figure 3B. **2nd Ranked Hit**: Close up views of the hAChE active site with bound **ar-curcumene** (carbon atoms in green), showing Ligand-Protein interactions points (yellow) at TYR 341, TYR 337, PHE 338.

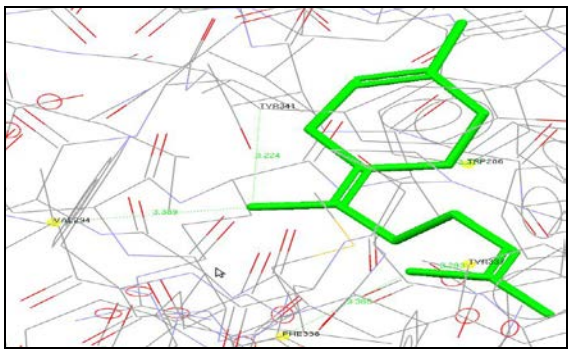

Figure 3C. 3rd Ranked Hit: Close up views of the HssAChE active site with bound **(Z)-α-bisobolene** (carbon atoms in green) showing Ligand-Protein interactions points (yellow) at TYR 341, TYR 337, TRP 286, PHE 338 and VAL 294**.**

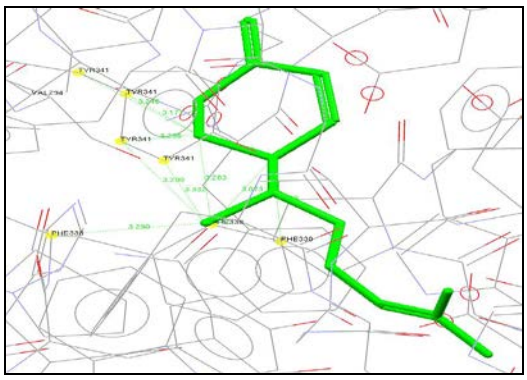

Figure 3D. 4th Ranked Hit: Close up views of the hAChE active site with bound **β-sesquiphellandrene** (carbon atoms in green), showing Ligand-Protein interactions points (yellow) at TYR 341, PHE 338, VAL 294.

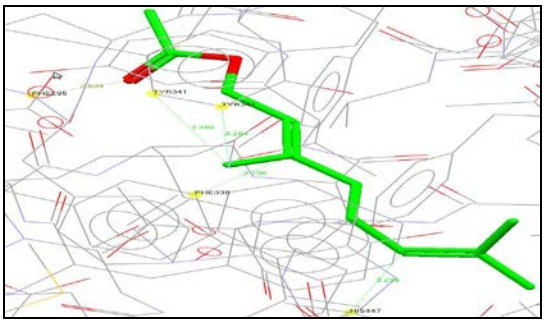

Figure 3E. 5th Ranked Hit: Close up views of the hAChE active site with bound **geranyl acetate** (carbon atoms shown in green, Oxygen atoms in red) showing Ligand-Protein interactions points (yellow) at TYR 341, PHE 338, PHE 295, HIS 447.

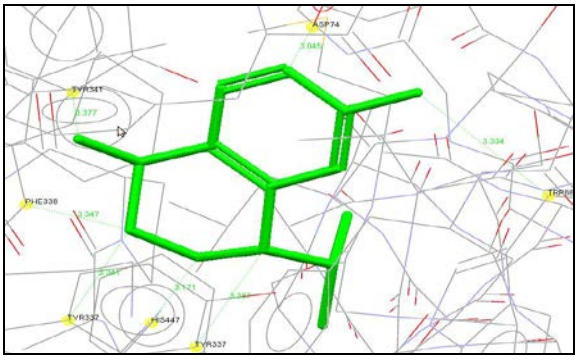

Figure 3F. 6th Ranked Hit: Close up views of the HssAChE active site with bound **trans-calamenene,** (carbon atoms in green) showing Ligand-Protein interactions points (yellow) at TYR 341, TYR337, PHE338, HIS 447, TRP86, and ASP74.

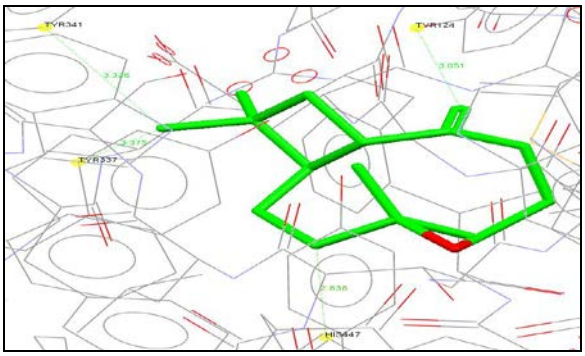

Figure 3G. 7nd Ranked Hit: Close up views of the hAChE active site with bound **caryophyllene oxide** (carbon atoms shown in green, oxygen atoms in red) showing Ligand-Protein interactions points (yellow) at TYR 341, TYR337, TYR124 HIS 447.

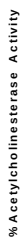

Figure 4. Acetylcholinesterase inhibitory activities of caryophyllene oxide and geranyl acetate. Values are mean +/- SD for n=3 replicate determinations. Eserine (IC_{50} = 0.67 nM) used as positive control.

Table 1. Ligand-protein interactions between top seven hit *A. citrodora* EO compounds and hAChE active site

	TYR 133	TYR 337	TYR 341	TYR 124	TRP 86	TRP 286	PHE 338	PHE 295	HIS 447	VAL 294	ASP 74
Huperzine A	X	X									
Donepezil		X	X		X	X					
β-Curcumene		X	X						X		
ar-Curcumene		X	X				X				
(Z)-α-Bisabolene		X	X			X	X			X	
***Trans*-Calamenene**		X	X		X		X		X		X
Caryophyllene oxide		X	X	X					X		
β-Sesquiphellandrene			X				X			X	
Geranyl acetate			X				X	X	X		

3.3. In vitro Experimental Studies

Three of the seven highest-ranking novel hits were commercially available to be evaluated against AChE *in vitro*. Initially, hits were screened against AChE on TLC plate. For these compounds with inhibitory potential, we performed a quantitative AChE inhibitory assay using the *in vitro* Ellman's method. The AChE inhibitor, eserine (IC_{50} 6.7 nM), was used as reference inhibitor. The resulting dose-response data were fitted using GraphPad Prism to estimate AChEI, IC_{50} values for two representative active hits, caryophyllene oxide (5.3 μM) and geranyl acetate (244.5 μM) (Figure 4). (Z)-α-Bisabolene was, apparently, not active.

4. Discussion

AChE is an attractive target for AD treatment because cholinergic deficit is a consistent symptom in the early stages of the disease [2]. Cholinesterases (ChEs) are type of α/β hydrolases that are responsible for the hydrolysis ACh into choline and acetic acid, an essential step for the restoration of the cholinergic neurons. These are divided into two sister enzymes as acetylcholinesterase (AChE; EC 3.1.1.7) and butyrylcholinesterase (BChE; EC 3.1.1.8) (Lewis *et al.*, 2002; McLaurin *et al.*, 2000. Bartolini *et al*., 2003)

Knowledge of the protein structure of AChE is essential for understanding its remarkable catalytic efficacy, the possibilities for rational drug design and developing therapeutic approaches. The structure of hAChE consists of a 12-stranded central beta-sheets surrounded by 14 α-helices. The protein is composed of five major domains: 1. catalytic site (Ser203, Glu334 and His447 residues); 2. anionic subsite (Trp86, Tyr130, Tyr337 and Phe338 residues); 3. acyl binding pocket, (Phe295 and Phe297 residues); 4. oxyanion hole (Gly121, Gly122 and Ala204 residues); 5. the peripheral anionic site (Asp74, Tyr124, Trp286, and Tyr341) (Habash *et al*., 2017; Lu *et al.*, 2011; Harel *et al*., 1996; Greenblatt *et al.*, 1999; Kryger *et al.*, 1999; Raves *et al*., 1997; Ambure *et al.*, 2014; Gupta *et al.*, 2011).

Literature review displayed promising evidence that supports the use of EOs for reversing cognitive and memory impairment of AD. Several studies regarding the cholinesterase inhibitory potential of EOs have been widely reported. The EOs of *Nigella sativa, Salvia officinalis, Acorus gramineus, Lavandula angustifolia, Melissa officinalis, Mentha piperita, Rosmarinus officinalis, Jasminum sambac, and Piper nigrum* have been proven for their putative effects *in vivo* studies together with clinical trial data (Adewusi *et al.*, 2010; Ryan and Byrne, 1988; Hansch and Deutsch, 1966; Miyazawa *et al.*, 1997; Perry *et al*., 1996 and Perry *et al.*, 2000).

A. citrodora EO also showed promising results in terms of AChE inhibition (Abuhamdah *et al*., 2015). In the present study, the essential oils of the fresh leaves of the plant, were obtained by hydrodistillation and chemically defined. The search for new AChE inhibitors, involved predicting interactions that occur between the ligand and the active site residues of the enzyme, and optimization of each single component of the oil using an algorithm scoring functions to choose the one that is most likely to be active with potential AChE inhibitory activity. Seven compounds were identified by using these molecular modeling studies. Four common interactions were detected for these active hits including: Tyr341 at peripheral anionic site, Tyr337, and Phe338 at anionic subsite domain and His447 catalytic anionic site domain respectively, and three major types of molecular interactions were exhibited: hydrogen bonds, hydrophobic interactions, and π-π interactions, visualized on the highest-scored poses.

The active site of AChE contains two main subsites, the esteratic subsite consisting in a histidine residue His447 and anionic subsites, of a tryptophan residue Trp84 corresponding to the catalytic machinery and the choline-binding pocket. Most non-alkaloidal AChE inhibitors, which include terpenes, flavonoids, and other phenolic compounds, seem to act as non-competitive inhibitors that bind to peripheral anionic sites (PAS) mainly represented by the residues Tyr70, Asp74, Try121, Trp279, and Tyr334. The seven hit compounds in our study exhibited a good docking score by interaction with His447, Tyr334, and Trp84, consistent with previous studies in the literature (Johnson and Moore, 2006). The information gained from these data indicated that the 3D structures of the active compounds and their molecular docking data to locate binding sites for ACh and inhibitors is a promising strategy to identify putative novel AChE inhibitors that are highly selective for their binding sites. The four new inhibitors in this case identified from *A. citrodora* will guide future experimental studies on these constituents and their potential for providing lead chemical structures for drug discovery. Chemical synthesis of the other three novel hits: β-curcumene, *trans*-calamenene, and β-sesquiphellandrene- as well as the isolated pure isomer β-/(Z)-α-bisabolene need to be available to further validate our model.

5. Conclusions

A molecular docking study predicting ligand-target interactions at a molecular level of *A. citrodora* essential oil components within human AChE, enables the identity of seven hit compounds forming favorable interactions at

the active site of the enzyme. *In vitro* evaluation of three available binding hits leads to discovery of two new active hits, namely caryophyllene oxide and geranyl acetate. Reflecting on our results, we can propose that these hits are promising candidates for future AD symptom-relieving drugs.

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Competing interests

The authors have declared no conflict of interest.

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Evaluation of *SIRT1* Gene Expression and rs3758391 C/T Polymorphism in Coronary Atherosclerosis

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Abstract

Recognition of disease-causing genes and susceptibility genes for coronary artery disease (CAD) will provide valuable data for prevention and control of CAD. The purpose of the present work was to investigate the association of *SIRT1* expression and its promoter single nucleotide polymorphism rs3758391 T/C with coronary atherosclerosis and its severity. Moreover, the current work aimed to explore the effectiveness of this polymorphism on *SIRT1* expression. The participants were categorized according to their angiographic results into CAD group with significant coronary artery atherosclerosis and non-CAD group with normal coronary artery. DNA was extracted followed by genotyping of *SIRT1* rs3758391 C/T. Extraction of total RNA was carried out, followed by PCR amplification of SIRT1 gene. CC genotype was prevalent in CAD group rather than control group (p=0.002) and conferred a greater risk of coronary atherosclerosis compared to those carrying CT+TT genotypes (OR: 3.41, CI: 1.81-6.42, $p < 0.001$). At allelic level, C allele was significantly prevalent in CAD patients rather than control group ($p < 0.001$). Additionally, *SIRT1* expression was significantly lower in CAD patients than in controls (p=0.001) and its expression reduced significantly in the CC genotype of both CAD and control groups compared to CT and TT genotypes (p=0.001). Moreover, there was an inverse correlation (rho = -0.23, p=0.02) between *SIRT1* expression of CAD patients and Gensini scores. In conclusion, *SIRT1* rs3758391 CC genotype might carry an increased risk of coronary atherosclerosis. However, *SIRT1* gene expression was inversely correlated with atherosclerosis severity suggesting its protective effects against atherosclerosis development.

Keywords:SIRT1 expression; SIRT1 rs3758391 C/T; genotyping; coronary atherosclerosis; Gensini score

1. Introduction

Coronary artery disease (CAD) remains typically the chief cause of mortality and morbidity in the whole world population (Simon and Vijayakumar, 2013). Despite persistent efforts in the management and prevention of this complex disease, it remains a big challenge to the scientists and health managers. It is expected that by the year 2020, this disease would remain as the most common and major threat to human life (Yusuf et al., 1998). In Egypt, mortality secondary to CAD is rapidly increasing. According to the latest WHO data, CAD deaths reached 21.73% of total deaths in Egypt (Ali et al., 2014) and reported a prevalence of 8.3% (Almahmeed et al., 2012).

CAD is considered as one of the complex diseases that are polygenic or multifactorial, produced by several genetic variants together with numerous environmental and lifestyle factors. It is characterized by long-term formation of atheromatous plaque, which culminates into atherothrombotic lesions resulting in tissue damage. Thrombosis and atherosclerosis are the major manifestations underlying CAD (Fawzy et al., 2017).

A huge number of laboratory researches, conducted within the past decade, have led to many achievements describing the genetic foundation of atherosclerosis (Libby et al., 2011). It has been recommended that more than a dozen of genes and genetic loci are related to CVD (Kathiresan and Srivastava, 2012).

Sirtuins (*SIRT1–SIRT7*) are family members of NAD^+-dependent protein deacetylases. Mammalian *SIRT1*, the best-recognized member of the sirtuin family, is an essential regulator of metabolism, cellular differentiation and senescence, cancer, and stress response (Poulose and Raju, 2015). Being a NAD^+-dependent enzyme, *SIRT1* regulates gene expression plans relating to cellular metabolic status (Zhang and Kraus, 2010). *SIRT1* is broadly expressed in various tissues with high expression in the vascular endothelium (Edirisinghe and Rahman, 2010).

SIRT1 plays an important role in decreasing atherosclerosis by intermediating several pathways. In addition to its function in enhancing NO production, *SIRT1* has anti-inflammatory capabilities in endothelial cells and even macrophages through decreasing the expression of numerous proinflammatory cytokines by meddling with the NF-κB signaling pathway (Stein et al., 2010 a). Furthermore, SIRT1 hinders the expression of lectin-like oxidized low-density lipoprotein (oxLDL) receptor 1 (Lox-1), a scavenger receptor for oxLDL,

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stopping the formation of macrophage foam cell, consequently delaying atherosclerosis (Stein et al., 2010 b). Besides, *SIRT1* activates ATP-binding cassette transporter A1 (ABCA1) transcription, which in turn mediates the HDL synthesis, reverses cholesterol transport and hence reduces the hazards regarding atherosclerosis and cardiovascular events (Li et al., 2007). The present body of evidence suggests that *SIRT1* dysregulation is involved in vascular dysfunction and the evolution of coronary artery disease. Therefore, *SIRT1* is considered as a hopeful target for new drugs for the management of CVD and related diseases (D'Onofrio et al., 2015).

Genetic variations such as single-nucleotide polymorphisms (SNPs) within the *SIRT1* gene have been linked with obesity (Zheng et al., 2012), type 2 diabetes (Han et al., 2015), hypertension (Zhong et al., 2015), and myocardial infarction (Cui et al., 2012; Yamac et al., 2019). Promoter region variants might be the cause of differential *SIRT1* expression, making people liable to certain pathologies (Nasiri et al., 2018; Mohtavinejad et al., 2015). SNPs within the *SIRT1* gene have been proposed to affect the messenger ribonucleic acid (mRNA) expression by modifying the binding ability of transcription factors or microRNAs (miRNAs) (Hu et al., 2015).

The management and evaluation of subjects with suspected CAD have been established on the noninvasive recognition of ischemia followed by using invasive coronary angiography (ICA) to approve the presence of luminal stenosis produced by atherosclerotic plaque (Schuijf et al., 2008). Coronary angiography is an essential tool for the quantification of CAD plaque burden in clinical practice (Roger et al., 2011). Despite being the golden standard for CAD diagnosis, ICA has numerous limitations (Yamashitaet al., 2010). Consequently, it has become a vital need to get an alternative noninvasive tool for precise diagnosis of CAD.

Identification of disease-causing genes and susceptibility genes for CAD as well as determining blood genetic markers for accurate noninvasive diagnosis of CAD are still a subject for research. Thus, the purpose of the current work was to investigate the association of *SIRT1* gene expression and its promoter single nucleotide polymorphism (SNP) rs3758391 T/C with coronary atherosclerosis and to explore the effectiveness of this polymorphism on *SIRT1* gene expression. Moreover, the current work aimed to evaluate the correlation between *SIRT1* expression and coronary atherosclerosis severity. Finally, the current work aimed to explore the value of *SIRT1* gene expression as a potential genomic predictor for CAD, validated by angiographic Gensini score.

2. Methods

2.1. Subjects and study design

The research ethics committee of Benha Faculty of Medicine approved the current clinical study. The current study was conducted according to World Medical Association (WMA) Declaration of Helniski (2008) and all study participants gave their written informed consents. Two hundred and fourteen subjects were invited to participate in the study, while fourteen refused to participate. The study subjects were different genders undergoing elective coronary angiography in the Cardiology Catheter Unit at Benha University Hospitals. According to coronary angiography, the subjects were categorized into CAD group (100 patients) with significant coronary artery atherosclerosis and non-CAD group (100 patients) with normal coronary angiograms to be a control group. Noteworthy, Significant coronary artery atherosclerosis was described as at least one particular major coronary artery having ≥70% or left main coronary artery having ≥ 50% luminal diameter stenosis (Reiber et al., 1989). Patients with heart failure, myocarditis, cardiomyopathies or those with history of coronary artery bypass grafting (CABG) and percutaneous coronary intervention (PCI) were excluded from the study. Exclusion criteria also included diabetes mellitus, severe renal or liver function defect, malignant tumor, inflammatory diseases or acute or chronic infections, and hematologic disorders.

2.2. Blood sampling

After an overnight fasting, 7.5 ml venous blood samples were withdrawn under complete aseptic conditions before undergoing coronary angiography. The particular collected blood samples were split up into three parts. First part (3 ml) was picked up in sterile tubes and left to clot for serum separation to be used for biochemical laboratory investigations, including total cholesterol, high-density lipoprotein (HDL), triglycerides (TG) and low-density lipoprotein (LDL). The second part (1.5 ml) was picked up in sterile glass vacutainer tubes containing ethylene- diaminetetraacetic acid (EDTA) then transferred into sterile Eppendorf tubes and stored at -20 ℃ to be used later for genotyping. The third part (3 ml) was collected in sterile EDTA- vacutainer tubes and the buffy coat layer containing peripheral blood mononuclear cells (PBMCs) was separated by Ficoll density-gradient centrifugation method using Ficoll Histopaque®-1077 (Sigma-Aldrich, U.S.A.) (Amos and Pool , 1976) following manufacturer's instructions. PBMCs were kept at -80 °C until later relative quantification (RQ) of *SIRT1* mRNA expression.

2.3. Coronary angiography

Elective coronary angiography was carried out for all patients included in the study via femoral artery using the Judkins technique or a radial approach. The angiographic characteristics were obtained from multiple views for both the left and the right coronary arteries. Two experienced cardiologists who were blinded to laboratory and clinical findings of the patients evaluated all the coronary angiograms. Gensini score assessed severity of CAD, which is grounded on the stage of the luminal stenosis and its regional importance (Gensini, 1983). Narrowing of the lumen was graded by Gensini score as follows: grade 1 (≤25% occlusion), grade 2 (26%–50% occlusion), grade 4 (51%–75% occlusion), grade 8 (76%–90A% occlusion), grade 16 (91%–99% occlusion) and grade 32 (total occlusion). Afterward, this primary score was multiplied by a component, which brings into consideration the significance of the location of the lesion within the coronary arterial tree. Gensini score was demonstrated as the summation of the scores for all 3 coronary arteries to estimate the total extent regarding CAD. Gensini score of 20 or more was considered severe CAD that was more or less equivalent to single stenosed

lesion of 70% or more within the proximal left anterior descending artery (Gensini et al., 1983).

2.4. Genotyping and SNP rs3758391 C/T analysis of SIRT1 gene:

DNA was extracted from peripheral blood using QIAmp DNA Blood Mini Kit supplied by (Qiagen, Germany) (Hedberg et al., 2015) in line with the manufacturer's instructions. Genotype of the samples was determined by TaqMan SNP Genotyping Assay (Applied Biosystems, U.S.A) specific for SIRT1 rs3758391 C/T according to the manufacturer's instructions. Briefly, the reaction contained 10 µL of 2X TaqMan® Universal Master Mix II (Applied Biosystems, U.S.A.), 1 µL of 20X TaqMan®Assay, 20 ηg/µL genomic DNA and RNase-free water up to 20 µL final volumes. The reactions were performed on StepOnePlus™ Real-Time PCR system (Applied Biosystems, U.S.A.). Genotypes were analyzed using StepOne Software v2.3 (Applied Biosystems, U.S.A.).

2.5. Analysis of human SIRT1 gene expression in PBMCs

RNA extraction

Total RNA was extracted from PBMCs using Direct-zol™ RNA MiniPrep supplied by Zymo Research, U.S.A. (Zhang et al., 2013) following the manufacturer's instructions. Nanodrop 2000 (Thermo Fisher scientific, USA) was used for the assessment of the quality and the quantity of the extracted RNA. Extracted RNA was then stored at -80°C for further processing.

Reverse transcription

Extracted RNA was reverse transcribed using high-capacity cDNA reverse transcription kit, supplied by Applied Biosystems, USA (Glenn et al., 2010) in line with the manufacturer's instructions. 1 µL of extracted RNA, completed to 10 µL by Nuclease- free H2O, was added to 10 µL of 2X RT master mix. The G-Storm Thermal Cycler (Gene Technologies, U.K.) program was 25°C for 10 minutes, 37°C for 120 minutes, 85°C for 5 minutes and 4°C for ∞. The cDNA product was diluted in 100 µL Nuclease-free H2O.

Real time PCR

PCR amplification of SIRT1 gene and the housekeeping gene GAPDH were carried out in separate PCR tubes using gene specific primers as follows: SIRT1: forward 5′- TGAGGCACTTCATGGGGTATGG-3′; reverse 5′- TCCTAGGTTGCCCAGCTGATGAA-3′ (Zhang et al., 2013); and GAPDH: forward 5′-GAAATCCCATCACCATCTTCCAGG-3′; reverse 5′-GAGCCCCAGCCTTCTCCATG-3(Li et al., 2014). The amplification reaction contained 12.5 µL of 2x SYBR® Green PCR master mix (Applied Biosystems, U.S.A.) (Romeiro et al., 2016), 2 µL of cDNA, 1 µL of the forward primer, 1 µL of the reverse primer and Nuclease-free H2O up to 25 µL final volumes. The PCR conditions were performed in the StepOne Real-Time PCR system (Applied Biosystems, USA) according to the following program: 95°C for 10 min, 40 cycles of 95°C for 15 Sec, anneal/extend temperature of (60°C for SIRT1 and 56°C for GAPDH) for 60 Sec.

Melting curve analysis using StepOne software (Applied Biosystems, USA) was performed to assess specificity of the amplification products. The level of SIRT1 mRNA in each sample was normalized to the mRNA level of GAPDH. Controls were chosen as the reference samples, and fold change of SIRT1 mRNA was determined by $2^{-\Delta CT}$ method (Schmittgen and Livak, 2008) using StepOne Software v2.3 (Applied Biosystems, U.S.A.).

2.6. Statistical analysis

The study data were analyzed using STATA/SE version 11.2 for Windows (STATA Corporation, College Station, Texas). Quantitative variables were expressed as mean ±SD and range. Qualitative variables were shown as number and percentage. Comparison of qualitative data was achieved using chi-square (χ^2) and Fisher's exact tests. The student t-test (t) and Mann-Whitney test (z) were used to compare two groups regarding parametric and non-parametric data respectively, while, Kruskal Wallis test ($\chi 2$) was used to compare more than two groups followed by post-hoc analysis using the Bonferroni method. The Odd's Ratio (OR) and 95% Confidence Interval (95% CI) were also considered. Spearmen correlations were used to correlate different variables. Receiver Operating Characteristics (ROC) analysis was performed to assess the diagnostic performance of *SIRT1* expression for atherosclerosis. The best cutoff point and the matching sensitivity and specificity, Positive Predictive Value (PPV), Negative Predictive Value (NPV) and Area Under the Curve (AUC) were analyzed. p value < 0.05 was considered significant.

3. Results

3.1. Baseline characteristics

A total of two hundred subjects who had undergone elective coronary angiography were included in this study. Demographic, laboratory and clinical data of the study subjects are presented in Table 1. Smoking was significantly higher in CAD patients, as compared with the control group (p=0.047). Additionally, there was a significant statistical decrease in the mean HDL-C level of the CAD group, as compared with the control group (p<0.001). Otherwise, no significant association with other studied parameters was detected.

3.2. Genotyping and allele frequency of SIRT1 rs3758391 T/C

The genotype distribution and allelic frequency of SIRT1 rs3758391 T/C among CAD and control groups are presented in Table 2. There was a statistically significant difference between both groups regarding genotype distribution of SIRT1 rs3758391 T/C (p value < 0.05). In the additive model, the frequency of rs3758391 CC vs. TT genotype was significantly higher in CAD patients than controls (OR: 3.34, CI: 1.50-7.47, p value < 0.05). In accession, in the recessive model, the frequency of rs3758391 CC vs. TT+CT genotypes was significantly higher in CAD group than controls (OR: 3.41, CI: 1.89-6.14, p value < 0.05). Moreover, the frequency of C allele was significantly higher in CAD group than controls (OR: 2.26, CI: 1.50-3.43, p value < 0.05).

Table 1. Demographic, laboratory and clinical characteristics of the study subjects

Qualitative variables		CAD patients (no.=100) %	Controls (no.=100) %	p
Sex	females	38	46	0.25
	males	62	54	
Smoking	Non smokers	46	60	0.047*
	Smokers	54	40	
HTN	Negative	35	45	0.15
	Positive	65	55	
Family history	Negative	80	89	0.08
	Positive	20	11	
Statin use	Negative	36	49	0.06
	Positive	64	51	

Quantitative variables	CAD patients (no.=100)		Controls (no.=100)		p
	Mean ±SD	Range	Mean ±SD	Range	
Age (years)	57.28±5.86	47-71	56.3±5.91	46-68	0.24
BMI (kg/m^2)	31.3±4.37	25.2-44.6	30.92±4.76	24.2-44.2	0.56
Cholesterol [mg/dl]	197.89±34.27	141-282	199.04±34.34	141-268	0.81
HDL-C [mg/dl]	36.12±3.72	27-43	39.66±4.98	30-51	<0.001*
TG [mg/dl]	165.16±54.35	70-287	159.02±32.44	90-235	0.33
LDL-C [mg/dl]	128.05±30.57	78-187.6	127.58±31.71	72-192	0.91

Variable	CAD patients (no.=100)	
	Mean ±SD	Range
Gensini score	36.05±27.68	6-122

*p<0.05 is significant

3.3. Genotyping and allele frequency of SIRT1 rs3758391 T/C

The genotype distribution and allelic frequency of SIRT1 rs3758391 T/C among CAD and control groups are presented in Table 2. There was a statistically significant difference between both groups regarding genotype distribution of SIRT1 rs3758391 T/C (p value < 0.05). In the additive model, the frequency of rs3758391 CC vs. TT genotype was significantly higher in CAD patients than controls (OR: 3.34, CI: 1.50-7.47, p value < 0.05). In accession, in the recessive model, the frequency of rs3758391 CC vs. TT+CT genotypes was significantly higher in CAD group than controls (OR: 3.41, CI: 1.89-6.14, p value < 0.05). Moreover, the frequency of C allele was significantly higher in CAD group than controls (OR: 2.26, CI: 1.50-3.43, p value < 0.05).

Table 2. The genotype distribution and allele frequency of *SIRT1* rs3758391 T/C among the study groups.

Genotype		**CAD (no.=100)**		**Controls (no.=100)**		χ^2	**p**	**OR (95% CI)**
		No.	**%**	**No.**	**%**			
Additive	TT	14	14.0	23	23	17.21	<0.001*	1.00
	CT	29	29.0	49	49	0.004	0.94	0.97 (0.43-2.81)
	CC	57	57.0	28	28	9.05	0.002*	3.34 (1.50-7.47)
Dominant	TT	14	14.0	23	23	2.69	0.10	1.83 (0.88-3.81)
	CT+CC	86	86.0	77	77			
Recessive	TT+CT	43	43.0	72	72	17.21	<0.001*	3.41 (1.89-6.14)
	CC	57	57.0	28	28			
Alleles	T	57/200	28.5	95/200	47.5	15.32	<0.001*	2.26 (1.50-3.43)
	C	143/200	71.5	105/200	52.5			

*p<0.05 is significant
OR: odd ratio
CI: confidence interval

3.4. Demographic and clinical characteristics according to different SIRT1 rs3758391 genotypes of the CAD and the control groups.

There were non-significant statistical differences regarding gender, age, smoking habit, hypertension, family history, statin use and BMI between different *SIRT1* rs3758391 genotypes among both CAD and control groups (P values > 0.05).

3.5. Laboratory parameters according to different SIRT1 rs3758391 genotypes of the CAD and the control groups.

There were non-significant statistical differences regarding mean cholesterol level, HDL-C level, TG level and LDL-C level between different *SIRT1* rs3758391 genotypes among both CAD and control groups (P values > 0.05).

3.6. Gene expression analysis

SIRT1 gene expression levels were analyzed and calculated as 2^-ΔCT. *SIRT1* normalized gene expression levels in the studied groups are presented in Figure 1. *SIRT1* expression levels were significantly (p=0.001) reduced in CAD patients with mean±SD of 0.53±0.41 compared to the control with mean±SD of 1.05±0.86. Moreover, *SIRT1* gene expression levels were significantly lower in CC genotype compared to TT and CT genotypes of both CAD and control groups Table 3.

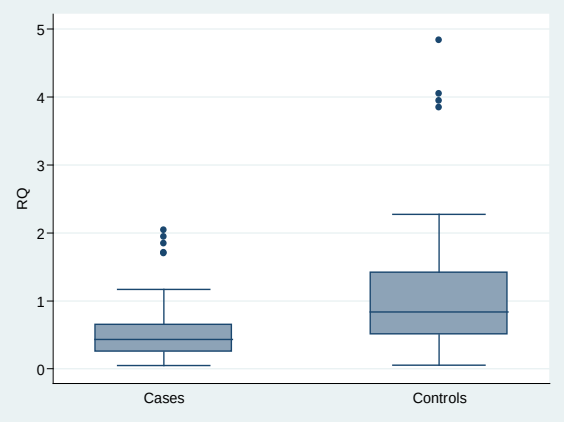

Figure 1. Normalized *SIRT1* gene expression levels among CAD and control groups.

Table 3. *SIRT1* expression levels according to different *SIRT1* rs3758391 genotypes of CAD and control groups.

Group	Genotype	*SIRT1* expression levels			Kruskal Wallis test (χ^2)	p
		No.	Mean ±SD	Range		
CAD group	TT	14	0.85±0.6	0.15-1.94	20.99	<0.001*
	CT	29	0.67±0.39	0.07-2.04		
	CC	57	†‡0.38±0.28	0.05-1.7		
control group	TT	23	1.39±0.89	0.05-4.04	17	<0.001*
	CT	49	1.14±0.92	0.08-4.83		
	CC	28	†‡0.63±0.47	0.09-1.68		

*p<0.05 is significant

†: Significant difference compared to genotype TT

‡: Significant difference compared to genotype CT

3.7. Gensini score analysis

Gensini score showed a significant inverse correlation with HDL-cholesterol (rho = -0.28, p=0.004) and a significant positive correlation with age (rho = 0.25, p=0.01). Moreover, Gensini score was significantly higher in males than in females (p=0.02) and even significantly higher in smokers than in non-tobacco users (p =0.04). Furthermore, Gensini scores were significantly higher in CC genotype, as compared with TT and CT genotypes of CAD group Table 4. On the other hand, non-significant associations or correlations with the other demographic, clinical, or laboratory data were detected.

Table 4. Gensini scores according to different *SIRT1* rs3758391 genotypes of CAD group.

Genotype	Gensini score			Kruskal Wallis test (χ^2)	p
	No.	Mean ±SD	Range		
TT	14	22.28±19.14	7-80	11.51	0.003*
CT	29	28.07±21.67	7-72		
CC	57	†‡43.49±29.96	6-122		

*p<0.05 is significant

†: Significant difference compared to genotype TT

‡: Significant difference compared to genotype CT

3.8. Correlation of SIRT1 gene expression with quantitative demographic and clinical data of patients and control

SIRT1 gene expression levels were correlated with quantitative demographic and clinical data as indicated in Table 5. *SIRT1* expression levels revealed a significant positive correlation with HDL-cholesterol in both CAD patients and even control group. On the other hand, it exhibited a significant inverse correlation with Gensini score in the CAD group. Otherwise, no other significant correlations were established.

3.9. Relation of SIRT1 expression levels with qualitative demographic and clinical data of CAD patients and control.

There were non-significant statistical differences in *SIRT1* expression levels regarding gender, smoking habit, hypertension, family history and statin use of both CAD and control groups (P values > 0.05).

3.10. Performance characteristics of SIRT1 expression (2^-ΔCT) as a predictor for atherosclerosis:

The best cut off values for 2^-ΔCT of the expressed *SIRT1* gene with the highest specificity, sensitivity, PPV and NPV for diagnosis of coronary atherosclerosis were determined and analyzed as shown in Table 6 and Figure 2. Using 0.0665 as a cut off value for 2^-ΔCt of *SIRT1* expression, the sensitivity and specificity of *SIRT1* to rule out coronary atherosclerosis was 76 % & 65%, respectively.

Table 5. Spearman's correlation of *SIRT1* expression levels with the quantitative demographic and laboratory data of CAD patients and control.

Group	Variable (no.=100)	Spearman correlation coefficient (rho; ρ)	p
CAD group	Age (years)	-0.02	0.80
	BMI (kg/m^2)	-0.09	0.38
	Cholesterol	-0.12	0.24
	HDL-C	0.30	0.003*
	TG	-0.08	0.45
	LDL-C	-0.16	0.11
	Gensini score	-0.23	0.02*
Control group	Age (years)	-0.14	0.17
	BMI (kg/m^2)	-0.11	0.27
	Cholesterol	-0.08	0.41
	HDL-C	0.22	0.03*
	TG	-0.09	0.38
	LDL-C	-0.11	0.28

*p<0.05 is significant

Table 6. Performance characteristics of *SIRT1* expression (2^-ΔCT) in predicting coronary atherosclerosis

Cut-off	0.665
Sensitivity (%)	76.00%
Specificity (%)	65.00%
PPV (%)	67.9%
NPV (%)	72.7%
Correctly diagnosed (%)	70.5%
AUC	0.7354

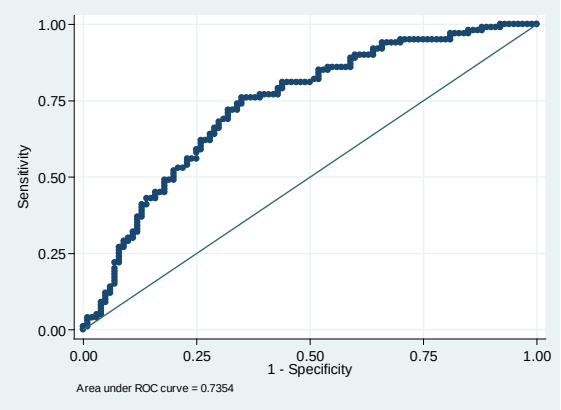

Figure 2. ROC analysis for *SIRT1* expression as a predictor for atherosclerosis

4. Discussion

CAD is a complex disease driven by numerous interactions of environmental and genetic factors. Identification of these interactions will offer valuable data for prevention and control of CAD. Important improvements have been made in identifying disease-causing genes and susceptibility genes for CAD (Abraham et al., 2014).

The International HapMap Project has recognized the presence of a single-nucleotide T/C polymorphism (rs3758391) in the identified p53-binding sequence of the *SIRT1* gene. The C allele of this polymorphism disturbs the mirror-image symmetry in the preserved nucleotides of the second half-site of the p53 binding sequence, raising the probability that this polymorphism may affect p53 binding affinity and hence impair *SIRT1* expression (Naqvi et al., 2010).

Regarding *SIRT1* rs3758391 T/C polymorphism, the current study revealed that CC genotype was more frequent in the CAD group (57%) than the control group (28%). Moreover, CC genotype conferred an increased risk of coronary atherosclerosis compared to those carrying CT+TT genotypes (OR: 3.41, CI: 1.81-6.42, p < 0.001). At the allelic level, C allele was significantly prevalent in CAD patients than the control group (71.5 % vs. 52.5 %) (OR: 2.27, CI:1.47-3.51, p < 0.001). These findings were consistent with a previous work on Iranian population, which revealed that rs3758391 CC genotype was more frequent in CAD patients in both additive (CC vs. TT) and recessive models (CC vs. TT +CT genotype) (Mohtavinejad et al., 2015). Furthermore, Mohtavinejad et al. (2015) concluded that the increase in the frequency of rs3758391 CC genotype might motivate the susceptibility of patients to CAD. In line with our results, they also reported that C allele was more prevalent in CAD patients than controls; however; the difference did not reach the borderline of statistical significance.

The present study revealed that there were no associations between rs3758391 SNP and total cholesterol, LDLc, HDLc and triglyceride levels. These results might not be representative of actual lipid profiles of patients involved in this study because most patients were under statin treatment. Nevertheless, these outcomes were consistent with a study conducted in Ashkenazi Jews (Han et al., 2015).

The current study demonstrated that Gensini scores were significantly higher in rs3758391 CC genotype carriers than in CT and TT carriers. In agreement with this finding, a previous study reported that carriers of the rs3758391 T allele had lower cardiovascular mortality risk among 1245 participants who were 85 years old or older (Kuningas et al., 2007).

The results of the current study revealed a statistically significant decrease in the expression level of *SIRT1* in PBMCs of CAD group, as compared with the control group. Several previous studies were in concordance with our results and reported the downregulation of *SIRT1* in PBMCs of CAD patients (Breitenstein et al., 2013; Li et al., 2016). Likewise, *SIRT1* expression was reported to be reduced in human atherosclerotic plaques in a study carried out by Gorenne et al. (2013). This finding was supported by a recent study, which reported that *SIRT1* inhibition induced the development of atherosclerotic plaque in ApoE–/– mice by increasing the expression of monocyte chemoattractant protein-1 (MCP-1) in addition to macrophage accumulation (Yang et al., 2017). Noteworthy, Chan et al. (2017) reported that LOX-1/oxidative stress signaling was induced and antioxidant enzyme activities were suppressed within CAD monocytes, suggesting the particular *SIRT1* role in protecting the cardiovascular system simply by means of its antioxidant, anti-inflammatory activities.

Based on the observations of the current study as well as the previous studies, the downregulation of *SIRT1* expression in CAD patients could be assigned to different factors. One of these factors may be the genetic variants of *SIRT1* gene. Increasing evidence suggested that *SIRT1* rs3758391 SNP impacts gene expression by decreasing p53 binding affinity, resulting in reduction of *SIRT1*

promoter activation and hence, down-regulation of *SIRT1* expression (Hu et al., 2015; Naqvi et al., 2010). Thus, rs3758391 in *SIRT1* gene promoter could be a functional SNP that might affect the transcription level of *SIRT1* gene.

The outcomes of the present work revealed that *SIRT1* expression correlated positively with HDL levels in both CAD patients and control groups. This finding was consistent with a study by Breitenstein et al. (2013) who suggested another contributing factor in the down-regulation of *SIRT1* expression in CAD patients. Breitenstein et al. (2013) proposed a regulatory role of HDL on *SIRT1* expression. They found that HDL of healthy subjects activated monocytes *SIRT1* expression considerably more prominently than HDL of patients with cardiac disorders, suggesting a signal mediating interaction between HDL and *SIRT1*. In addition, they suggested that Paraoxonase 1 (PON1), an HDL-associated antioxidant enzyme, is essential to stimulate *SIRT1* expression. Thus, reduced *SIRT1* expression levels in CAD patients appeared to be linked to reduced HDL levels and HDL dysfunction.

The current study revealed that *SIRT1* expression significantly reduced in CC genotype (the predominant genotype among the CAD group) as compared with CT and TT genotypes. This observation was supported by Hu et al. (2015) who demonstrated a significant association between the rs3758391 TT genotype and a higher *SIRT1* expression.

This study revealed that *SIRT1* expression levels in PBMCs of CAD patients showed a significant inverse correlation with Gensini score suggesting its role as an anti-atherogenic gene. Consistent with our results, de Kreutzenberg et al. (2010) reported that *SIRT1* expression was negatively correlated with subclinical atherosclerosis evaluated by carotid intima - media thickness. Conversely, our results were in contrast with Li et al. (2016) who did not detect a relationship between *SIRT1* expression, and the severity of coronary lesions evaluated by the Syntax score in patients with CAD and T2DM, recommending that *SIRT1* role in preventing atherosclerosis in diabetic patients be clarified. This inconsistency could be assigned to different measures of patient selection and to different amplification techniques used. Their study included diabetic patients and used semi quantitative PCR in evaluating *SIRT1* expression.

To estimate the diagnostic performance of *SIRT1*gene expression for atherosclerosis, the best cut off point for 2-ΔCt of the expressed gene and the matching sensitivity and specificity, PPV, NPV for prediction of coronary atherosclerosis were analyzed. The results showed that *SIRT1* expression had a good capability (Šimundić, 2009) in predicting CAD, suggesting that *SIRT1* expression might be of significant value in the prediction of CAD. Consistent with our results, He et al., 2019 reported that serum SIRT1 might play a predictive role in screening high- risk coronary plaques. The limitations of the current study were the relatively small sample size and the inability to study the racial and the ethnic effects because the study participants were all Egyptians.

5. Conclusion

SIRT1 rs3758391 CC genotype might confer an increased risk of coronary atherosclerosis. Additionally, *SIRT1* gene was down regulated in PBMCs of CAD patients and its expression was inversely correlated with atherosclerosis severity suggesting its protective effects against atherosclerosis development. Moreover, the variation in *SIRT1* expression was related to the presence of promoter SNP rs3758391 providing awareness to the mechanisms by which common non-coding genetic variants affects the target gene expression. Furthermore, *SIRT1* expression might be a valuable adjunct in diagnosing CAD.

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Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Chronic Effects of Lead Exposure on Oxidative Stress Biomarkers in Feral Pigeon (*Columba livia*) from Smelter Area in Kosovo

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Abstract

The effects of lead exposure on oxidative stress biomarkers in feral pigeon (*Columba livia*) from Mitrovicë town (situated in close vicinity of ormer lead and zinc smelter) were studied through the use of blood Pb (PbB) levels, d-aminolevulinic acid dehydratase (δ-ALAD) activity, level of plasma δ-Aminolevulinic acid (ALA), Glutathione (GSH), Malondialdehyde (MDA), Uric acid (UA), Urea (U), and creatinine (CR). Lead levels in pigeons from Mitrovicë were significantly elevated (P<0.001) compared to the control. Elevated PbB in pigeons from Mitrovicë was also accompanied by significantly inhibited (P<0.001) δ-ALAD activity in blood, significantly decreased levels of GSH and MDA (P<0.001; <0.05), and significantly elevated levels of ALA, UA, CR (P< 0.001) compared to the control. Negative correlation was observed between PbB and δ-ALAD activity (r= -0.409; P<0.05) in birds in Mitrovicë. This study provides important evidence about the chronic effects of lead on the analyzed oxidative stress biomarkers. In addition, the study proves that the pigeons in Mitrovicë remain chronically exposed to harmful effects of lead, and that the close vicinity of the former smelter "Trepça" still represents a source of exposure to lead for the health of biota and humans.

Keywords: lead level, oxidative stress biomarkers, δ-ALAD, feral pigeon, Mitrovicë.

1. Introduction

The long-term pollution activities from smelting factories may disturb or destroy ecosystems, thereby making them less suitable for wildlife. In fact, these polluted sites may produce bioavailability and increased levels of toxic compounds such as heavy metals, which affect multiple generations of the population as a result of their extended bioavailability (Dimitrov *et al.,* 2016).

Heavy metals are highly reactive and often toxic at low concentrations. They may remain in the environment for years, posing long-term risk to life long after pollution sources have been removed (Gall *et al.,* 2015). Therefore, the use of monitoring in pollution assessment is of paramount importance.

Lead contamination is a global problem affecting the health of different bird species worldwide (Haig *et al.,* 2014). In this sense, lead (Pb) is a naturally occurring but nonessential element that is highly toxic at elevated concentrations having the potential to affect most body systems and the health of animals. Moreover, this toxic metal is considered one of the most significant threats to several species (Haig *et al.,* 2014; Wiemeyer *et al.,* 2017; Isomurso *et al.,* 2018; Helander *et al.,* 2019; Ecke *et al.,* 2017).

Birds are the most studied and probably the most affected species to lead poisoning (Pain *et al.,* 2019; Plaza and Lambertucci, 2019). In particular, wild birds have been disclosed to be useful bio-indicators because they are sensitive to pollutants and are important structural components of the ecosystem (Kekkonen *et al.,* 2012; Matheo-Thomas *et al.,* 2016).

Negative impacts of pollution from mining and smelting complexes have been observed in wild bird species for decades. Although the technological advances and legislation have resulted in reduced impact from metal industries in terms of metal emissions, polluted soils may still constitute a major source of heavy-metal exposure for wild birds, even if the atmospheric deposition declines (Berglund *et al.,* 2010; Elezaj*et al.,* 2013).

In the last years, the number of studies evaluating the effects of heavy metals on oxidative stress biomarkers in free-living birds exposed to heavy metals under natural conditions has increased (De la Casa *et al.,* 2015; Espin *et al.,* 2014a, 2014b; Koivula *et al.,* 2011; Martinez-Haro*et al.,* 2011; Rainio*et al.,* 2013). However, inter-specific differences due to different tolerance to metals have been found by some researchers (Espin *et al.,* 2014a, 2015; Hernandez-Garcia, 2010; Koivula and Eva, 2010), and the specific resistance of species to metals is still unclear for many birds (Espin *et al.,* 2016).

From this perspective, it is essential to use some biomarkers to detect oxidative stress in birds. This is mainly due to the fact that different antioxidants are involved in the protection against reactive oxygen species

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(ROS) through close interaction between them, and that the antioxidant defense may respond differently depending on the species (Berglund *et al.,* 2007; Constantini and Verhulst, 2009; Koivila and Eva, 2010).

The concentrations of lead produce harmful effects on bird's health like inhibition of enzymes that are important for metabolic functions (Espín *et al.,* 2015). One of the prime targets of lead toxicity is the heme synthesis pathway. In this pathway, δ-ALAD is the most sensitive and specific biomarker of the low lead (Pb) exposure in humans and wild animals including bird species (Elezaj *et al.,* 2013).

In this sense, ALA-D inhibition results in correlated decrease in hem production of hemoglobine and accumulation of Aminolevulinic acid (ALA), which can be oxidized to generate ROS. Consequently, enhanced lipid peroxidation and DNA damage may occur. The δ-ALAD can serve as a valuable biomarker of oxidative stress in hematological system exposed to lead and act also as a biochemical indicator of lead exposure (Gurer-Orhan *et al.,* 2004).

Recent studies have also explained the role of antioxidants in wild birds, with particular attention to low-weight non-enzymatic molecules such as Glutathione (GSH) and Uric acid (UA) (Constantini *et al.,* 2008; Cohen and McGraw, 2009; Perez-Rodrigez *et al.,* 2009; Koivula and Eva 2010; Sánchez-Virosta *et al.,* 2019; Dolan *et al.,* 2017).

Lipids are the most involved class of bio-molecules of the biological targets of oxidative stress. Lipid peroxidation induces a number of secondary products, among which Malondialdehyde (MDA) is the major and most studied product of saturated fatty acid peroxidation. MDA is highly toxic molecule and should be considered as more than just a marker of lipid peroxidation. Its interaction with DNA and proteins has often been ascertained as potentially mutagenic and atherogenic (Del Rio *et al.,* 2005).

The aim of this study was to assess potential effects of blood Pb on oxidative stress biomarkers in free-living feral pigeons (*Clombalivia*) from Mitrovicë town, situated to a close vicinity of former smelter "Trepça", North East of Kosovo, and Lukinë village-rural area. For this purpose, the study analyzes the blood Pb level (PbB), and some biomarkers of oxidative stress such as δ-ALAD activity, levels of ALA, GSH, MDA, UA, U, and CR.

2. Material and Methods

2.1. Study area

Mitrovicë (Fig.1) had the largest metallurgic and mining complex "Trepça" in Europe, which commenced activities in 1939 with the extraction of lead, cadmium, and zinc. Many industrial plants existed in the complex such as lead smelter, fertilizer production plant, refinery, battery factory, zinc electrolysis facility, and a sulfuric acid plant. The significant amount of heavy metal pollutants was released to the surrounding area, including residential areas, associated with biota and human health risk (Borgna *et al.,* 2009).

The study data collected from the close vicinity of Mitrovicë show that the average content of Pb in soil is 20-fold higher than the European median; Cd 11-fold, Hg 5.5-fold, As 4.6-fold, Zn 4.2, and Cu 3.2-fold higher.

In the narrower vicinity of Mitrovicë and Zveçan, the content of the aforementioned elements is even higher than the intervention values, according to the New Dutch list, and were exceeded in 152 km 2 of the investigated area. (Šajn *et al.,* 2013).

Lukinë village is located in southern west part of Kosovë, 120 km away from Mitrovicë town (Fig. 1).

Figure1. Localities ithe Map of Republic of Kosovo

2.2. Experiments

Specimens of Feral pigeons (*Columba livia*), a total of 20 birds of both genders from each locality were collected in March 2017from Mitrovicë and Lukinë. In urban areas, this species is sedentary, forming discrete flocks which remain faithful to specific feeding and roosting areas. For this reason, a relatively small sample of birds is expected to reflect any variation in metal exposure at the local level.

In laboratory setting, blood samples were collected by heparynized syringes directly from heart. Blood Pb level (PbB) was determined with atomic absorption spectrometry with an atomic absorber- Varian spectra AA 640Z Zeeman AAS, equipped with a GTA 100 graphite furnace (Varian, USA) and PSD 100 auto sampler (Varian, USA).

Erythrocyte δ-Aminolevulinic acid dehydratase (δ-ALAD) activity was measured according to the CEC standardized method. The level of δ-Aminolevulinic acid (δ-ALA) in plasma was determined by the spectrophotometric method (Berlin and Schaller, 1974). The level of malondialdehyde (MDA) in plasma was determined by the spectrophotometric method (Uchiama and Michara, 1978). Buetler's method was used to determine the total level of reduced glutathione (GSH) in the blood (Buetler *et al.,* 1963). The level of plasma uric acid (UA), urea (U) and creatinine (CR) was determined spectrophotometrically using Bio-La-Tests (Humana-Germany).

2.3. Data analysis

Data analysis of the results was carried out with Sigma stat 32 programs (2004 STAT) Software. For each continuous variable, a distribution form was determined, and significant differences between means were checked by Student's t test. Parson's correlation test was performed to examine the relationship between δ-ALAD activity and

Pb concentrations; and between δ-ALAD activity and other oxidative stress biomarkers. A value of p<0.05 was considered statistically significant. Data were expressed as means ± SD.

3. Results

Our results of blood Pb level (PbB), δ-aminolevulinic acid dehydratase activity (δ-ALAD), level of ALA, MDA, GSH, UA, U and CR in individuals of Feral pigeon (*Columba livia*) from Mitrovicë and Lukinë are presented in the table and figures (table 1 and figures 2 and 3).

Table1. Blood Pb level (PbB), δ-aminolevolunic acid dehydratase activity (δ-ALAD), level of (ALA), Glutathione (GSH), Malondialdehyde (MDA), Uric acid (UA), Urea (U), and Creatinine (CR), in Feral pigeon (*Columba livia*), in Lukinë and Mitrovicë.

Parameters	No\Loc	Lukinë (A)	Mitrovicë (B)	Significance *P*<
PbB (µg/dL)	20	4.7 ± 1.1	17.1 ± 10.3	A:B; *P*<0.001
ALA-D (U/LE)	20	45.8 ± 11.5	17.6 ± 12.6	A:B; *P*<0.001
ALA mmol/L	20	0.11 ± 0.09	0.66 ± 0.41	A:B; *P*<0.001
GSH (g/%)	20	35.4 ± 12.7	22.7 ± 3.9	A:B; *P*<0.001
MDA (µmol/L)	20	2.7 ± 1.4	1.04 ± 0.6	A:B; *P*<0.001
UA (µmol/L)	20	215 ± 65	326 ± 85	A:B; *P*<0.001
U (mmol/L)	20	0.5 ± 0.3	0.6 ± 0.2	NS
CR (mmol/L)	20	34.6 ± 4.0	39.1 ± 4.0	A:B; *P*<0.01

Note: The results are expressed as means X and standard deviation SD. NS-non significant.

The blood Pb level (PbB) in the Feral pigeons from vicinity of former lead and zinc smelter "Trepça"in Mitrovicë was significantly higher (*P*<0.001) with values of (17.1+10.3 µg/dL), compared with the rural area of Lukinë (4.6+11.2 µg/dL).

Blood δ-ALAD activity was inhibited (*P*<0.001), level of MDA and GSH significantly decreased (*P*<0.05; <0.001), while ALA and UA values were significantly higher (*P*<0.001) in pigeons from Mitrovicë compared with controls, while CR was significantly (*P*<0.01) higher.

There was negative correlation established between blood lead level and δ-ALAD activity (r= -0.409; *P*<0.05) in birds of Mitrovicë.

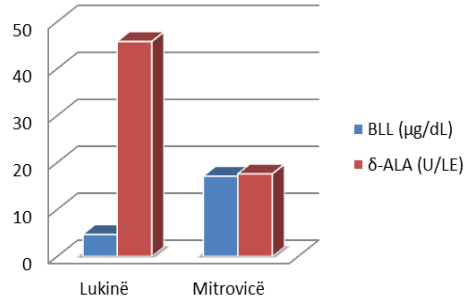

Figure 2. Blood lead level (PbB) and δ-ALAD activity in feral pigeons of Lukinë and Mitrovicë.

The level of plasma ALA in birds from Mitrovicë was 6.0 fold higher compared with that in Lukinë.

The level of MDA in plasma of pigeons from Lukinëis was significantly higher compared with pigeons in Mitrovicë (2.7 ±1.4; 1.0 ± 0.6; respectively; *P*< 0.01). The level of plasma MDA in birds from Mitrovicë is 1.7 times lower compared with the plasma MDA of pigeons in Lukinë.

A level of plasma GSH in pigeons from Mitrovicë (22.7 ± 3.9) was significantly lower (*P*<0.001) compared with birds from Lukinë (35.4 ± 12.7).

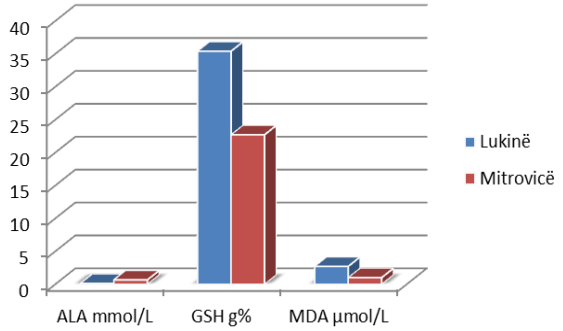

Figure 3. The level of ALA, GSH and MDA in feral pigeons of Lukinë and Mitrovicë.

Plasma uric acid level (UA) in Mitrovicë (326 ± 85) is significantly higher compared with the plasma UA of birds in Lukinë (251 ± 65; *P*<0.001), while the urea (U) in pigeons from Mitrovicë (0.6 ± 0.2), although not significantly, is higher than in plasma (0.5± 0.3) of pigeons from Lukinë.

The plasma level of creatinine (CR) in pigeons from Mitrovicë (39.1 ± 4.0) is significantly higher (*P*< 0.01) compared with pigeons in Lukinë (34.6 ± 4.0).

4. Discussion

The results of Pb level in the blood of Feral pigeons (Columba livia) from Mitrovicë (17.1+10.3 µg/dL) are significantly higher (P<0.001) compared with the control group of Lukinë pigeons (4.6+11.2 µg/dL). This confirms the presence of the risk of lead contamination in this area not only for the pigeons but also for the humans and other animal species.

Compared with our earlier results (Elezaj et al., 2008, 2013) about PbB in pigeons in Mitrovicë from 2000 to 2003 (the year when the smelter was closed down), these results are 4.9 times lower, 5.5 (2001); 2.6 (2002); 4.0 (2003) and 8.7 times lower than in 2012. The intermittent analysis of blood Pb levels (from 2000-2017) in the free-living pigeons from vicinity of lead and zinc smelter in Mitrovicë revealed the progressive decrease of blood lead levels in pigeons of Mitrovicë over the seventeen years (Elezaj et al., 2008, 2013; our data, 2017).

Our results of blood Pb level in birds from Mitrovicë are consistent with the results of Vanparys (Vanparys et al., 2008) who in the blood of great Tit (Parus major) from polluted areas collected 500-1000mcaused by a non-ferrous metallurgic smelter in the south Antwerp (Belgium). Vanparys found significantly higher blood Pb levels (5.8 times higher) compared with reference.

Coeurdassier (Coeurdassier et al., 2012), established higher levels of Cd and Pb in the blood and erythrocytes of European Black birds (Turdus merula) caught up from a contaminated site-smelter of Metaleurop Nord, North France.

Our results of blood Pb level in pigeons from Mitrovicëare are consistent with the results of Cai and Calisi (Cai and Calisi, 2016) who in pigeons (Columba livia) from the Soho/Greenwich Village neighborhood revealed highest levels of lead in their blood (X=23.121µg/dL), followed by pigeons from Lower Manhattan/Lower East Side (X=22.708 µg/dL) and Upper West Side (X= 19.957 µg/dL). Correlation data revealed that levels of blood of pigeons from the Manhattan neighborhoods positively correlate with the rates of children in the same neighborhoods with the elevated levels of blood lead (r = 0,647, N=8, P=<0.042).Whatever sources may be causing lead toxicity in their subjects, there exists a parallel between high blood levels in the pigeons sampled and high rate of children with blood Pb levels of 10µg/dL or more in NYC neighborhoods. They (Cai and Calisi, 2016) provide a powerful example of how monitoring pigeon biology may help us to better understand the location of prevalence of lead with the aim of providing greater awareness and devising prevention measures.

The results indicate a decrease of Pb exposure in the last 17 years in Feral pigeons (Columba livia) and House sparrows (Passer doemsticus) free-living in Mitrovicë town (Elezaj et al., 2013; Millaku et al., 2015), which could be explained by the closure of lead and zinc smelter "Trepaça" in Mitrovicë in the year 2000.

Our results of δ-ALAD activity in the blood of feral pigeons from Mitrovicë (inhibited 62.4 % v-s reference) explain the sensitivity of this biomarker of lead exposure. The results also confirm that this inhibition of δ-ALAD activity occurs due to the prevailing high level of lead pollution in this area.

The results are consistent with our former results (Elezaj et al., 2012) found in feral pigeons from the same area in 2012 when the δ-ALAD activity was 94.9% inhibited v-s reference (1.2 ± 0.9; 23.4 ± 3.0 U/LE respectively), while blood lead level was 149 ± 60 µg/dL v-s 5.4 + 0.6) µg/dL in the reference. In 2013, Elezaj (Elezaj et al., 2013) in the farm pigeons from Mitrovicë, established 77.8 % inhibition of δ-ALAD activity of pigeons from Mitrovicë v-s reference (5.2 ± 2.8; 23.4 ± 3.0 U/LE), while blood lead level was 50 ± 25; 5.4 ± 0.6 µg/dL respectively. The inhibition of blood δ-ALAD activity (61%) was marked also in the feral pigeons from courtyard of ferronickel smelter in Drenas (Elezaj et al., 2011).

Due to lead exposure across the observed range of Pb concentrations (0.01–0.34 µg/g), in nestling of Golden Eagle (Aquila chrysaetos), in western United States (California, Idaho, Oregon, Wyoming), Herring (Herring et al., 2020), found 68% reduction in delta-aminolevulinic acid dehydratase (δ-ALAD) activity.

Our results of inverse correlation established between blood Pb level (PbB) and δ-ALAD activity (r=-0.409, P<0.05) in feral pigeons from Mitrovicë explain the effect of the continuous increase of lead in blood in the continuous increase inhibition of activity of this oxidative stress biomarker.

These are consistent with the results of Elezaj's studies (Elezaj et al., 2011) that established inverse relationship between Pb concentrations in femur bone (51 µg/g d.w) and tibia (35.9 µg/g d.w.), v-s blood δ-ALAD activity (r=-877, P<0.001; r=-787, P<0.01 respectively) in free-living feral pigeons (Columba livia) in the courtyard of Ferronickel smelter in Drenas, Kosovo.

Correlation between delta-aminolevulinic acid dehydratase (δ-ALAD) activity and whole-blood lead (Pb) concentration in nestling Golden Eagles (Aquila chrysaetos) is also established in the western United States (California, Idaho, Oregon, Wyoming). Delta-aminolevulinic acid dehydratase activity declined 68% across the observed range of (0.01–0.34 lg/g) Pb concentrations (Herring et al., 2020).

Based on the collective data of the Federal National Resource Damage Assessment regulations (NRDA; regulation 43 CFR 11.62) have set the Pb-poisoning injury level in wildlife at >50%, decrease in blood δ-ALAD activity compared with unexposed reference animals, which again include birds (Van der Merwe et al., 2011). Taken together, these findings suggest that the recommendations of the acceptable lower threshold-limit for blood lead in feral pigeons and different free-living bird species require reexamination.

Our results of higher inhibition index of δ-ALAD activity (38.4%) can be explained by the fact that negative inversion between blood lead level and blood δ-ALAD activity above > 20 µg/dL disconnects the progressive linearity.

The results of higher Aminolevulinic acid (ALA) level (6.0-fold higher), as a precursor to δ-ALAD in the plasma of Feral pigeons from Mitrovicë town are consistent with the results of Costa (Costa et al.,1997), who in lead exposed workers established the positive linear relationships between plasma ALA level and blood lead level. Our data are in line with free radical hypothesis for lead poisoning, where ALA distribution to and accumulation in several organs may trigger oxidative stress response, not only in the case of lead exposed workers (Costa et al.,1997), but also in wild animals (birds), in conditions of environmental pollution with lead.

The correlation data between levels of Aminolevulinic acid (ALA-P), PbB, and δ-ALAD activity to our knowledge so far have not been investigated not only in wild pigeons but also in other birds. Thus, this draws attention to the fact that the amount of plasma ALA may have contributed to causing oxidative stress in individuals of these populations through the formation of free radicals, similar to causing oxidative stress in workers exposed to lead poisoning.

According to our data of the measure of the values of ALA in the feral pigeons, it turns out that the obtained results of this biomarker of oxidative stress in the feral pigeons are among the first to be investigated in these species.

It is important to explain that many metals are capable of generating Reactive oxygen species (ROS) and include oxidative damage and may therefore lead changes in oxidative regulation (Rianio et al., 2013). Oxidative state (in terms of GSH-reduced and GSH/GSSG-oxidized ratio), did not vary among species, suggesting that different species may employ different antioxidant pathways to achieve the same oxidative state. According to them, the effects on oxidative status observed in one species cannot be generalized to the other ones (Rainio et al., 2013). Of all the biomarkers that we examined, only glutathione peroxydase (GP) and glutathion-S-transferase (GST) activities in blue tits and GSH level in pied flycatchers

showed any association with metal contamination (Rainio et al., 2013).

Our results of lower concentration (34 %) of GSH in plasma of feral pigeons from smelter vicinity compared with the reference pigeons are consistent with the results of Sugawara (Sugawara et al., 1991), who in the erythrocytes of workers exposed to lead established lower concentration of GSH. In addition, previous studies have also observed that Pb concentrations above 10-15 ug/dL (or even lower) may produce inhibition of antioxidant enzymes, depletion of GSH, and induction of Thiobarbituric acid reactive substances (TBARS) in erythrocytes of Griffon vultures and Eagle owls (Espin et al., 2014).

Our results of lower level in the plasma of MDA in feral pigeons from smelter vicinity are consistent with the results of Dobrakovsky (Dobrakovsky et al., 2017), who in occupationally workers exposed to lead for 36 to 44 days (with blood Pb level 49.1 + 14.1 ug/dL at the end of study) established no change of malondialdehyde (MDA) level and activities of catalase (CAT) and superoxide dismutase (SOD). According to them, short-term exposure to lead induces oxidative stress associated with elevated level of lipid peroxides (LPH), but not MDA.

In addition, our results of higher level of plasma uric acid, urea, and creatinine in the birds from smelter vicinity are consistent with results of Hamidipour (Hamidipour et al., 2016), who in blood of Japanese quails exposed to 0.4 mg/kg diet of “Lead Acetate” for 21 days found significant increase of enzymes aspartate aminotransferase (AST), alanine aminotransferase (ALT), lactic acid dehydrogenase (LDH), glucose, creatinine, and uric acid, compared with the control group.

Additionally, creatinine concentration in blood plasma is nearly constant in natural conditions. Excessive amounts are also excreted through glomerular filtration. Its sharp increase in plasma is a sign of impaired glomerular filtration and renal dysfunction (Newman and Price, 1999).

The increase of plasma creatinine may be due to the adverse impact of lead or other metals on kidney function and glomerular filtration in birds from smelter vicinity. Uric acid rise may be due to the adverse effect of lead on kidney function. Because birds are uricotelic, measurements of uric acid are more valuable than creatinine and urea to asses kidney function (Khaki et al., 2011).

The level of uric acid, in addition to being a waste product, is also considered a biomarker for many physiological characteristics in vertebrates, including birds, because it cannot be broken down in vivo. It is the most dominant antioxidant defense system for birds and can be linked to their ability to cope with the heightened metabolic ROS production throughout their lives (Carro et al., 2012).

Urea serves an important role in the metabolism of no nitrogen containing compounds in animals. Urea is present in very small amounts in avian plasma, and plasma urea concentration has traditionally been considered an inappropriate parameter to evaluate renal function in birds (Lumeij and Remple, 2007).

Creatinine is reportedly of questionable value for evaluating renal function in birds because birds excrete creatine before it has been converted to creatinine. Some data suggest that creatine and creatinine may act as precursors of food mutagens and uremic toxins. Recent identification and purification of many of the enzymes involved in creatinine metabolism have just opened the door to wide variety of biological, physiological, as well as clinical investigations and applications (Lumeij and Remple, 2007).

5. Conclusion

The results of this study support the effects of lower levels of PbB on analyzed oxidative stress biomarkers. Additionally, the results confirm that feral pigeons in Mitrovicë remain chronically exposed to harmful effects of lead levels, and the close vicinity of smelter "Trepça" still poses a threat for the health of humans and biota.

Conflict of Interest

The authors have no conflicts of interest to declare.

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Characterization of the Complete Chloroplast Genome of *Blepharis ciliaris* (Acanthoideae, Acanthaceae)

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Abstract

The complete chloroplast genome of *Blepharis ciliaris*, medicinal and endangered plant in Saudi Arabia was sequenced and characterized. NOVOPlasty was used to assemble the complete chloroplast genome from the whole genome data. The cp genome of *B. ciliaris* is 149, 717 bp in length with GC content of 38.5% and has a circular and quadripartite structure; the genome harbors one pair of inverted repeat (IRa and IRb 25, 331bp each) separated by large single copy (LSC, 87, 073 bp) and small single copy (SSC, 16, 998 bp). There are 131 genes in the genome, which include 79 protein-coding genes, 30 tRNA and 4 rRNA; 113 are unique while the remaining 18 are duplicated in IR regions. The repeat analysis indicates that the genome contained all types of repeats with palindromic occurring more frequently; the analysis also identified a total number of 91 simple sequence repeats (SSR) of which the majority are mononucleotides A/T and are found in the intergenic spacer. This study reported the first cp genome of the genus *Blepharis* and provides resources for studying the genetic diversity of *B. ciliris* as well as resolving phylogenetic relationship within the core Acanthaceae.

Keywords: Acanthaceae; *Blepharis ciliaris;* chloroplast genome; SSR

1. Introduction

Blepharis ciliaris (L.) B. L. Burtt. is a member of the tribe Acantheae (Acanthaceae). The species is known to be distributed in Saudi Arabia, East Africa, East Pakistan and Egypt (Kamal and Abdul, 1956). The plant is used as fodder for ruminant animals particularly camel and sheep. The plant seeds (powdered) are used as an antibacterial on boils, wounds and sores. The seed of the plant is being used to treat cough and has diuretic, aphrodisiac and antibacterial activity (Deshpande, 2006). In addition, the charcoal from the root is used to improve eye vision and treat sore eyes (Boulos, 1981; Tackholm, 1974). Despite the endangered nature and uses in traditional medicine of the species, the complete chloroplast genome of the species was not characterized until this study.

Comparison of complete chloroplast genome provides very informative information for the reconstruction of phylogeny and resolving evolutionary relationship issues at various taxonomic levels (Shaw *et al.,* 2007; Mardanov *et al.,*2008; Moore *et al.,* 2010; Park *et al.,* 2017; Sun *et al.,*2017). This is as a result of the conservative nature of the chloroplast genome (Wolfe *et al.,* 1987); this conservative nature is because the plastome evolves about half the rate of other genomes like the nuclear genome (Jansen *et al.,* 2005; Walker *et al.,* 2014). However, rearrangements in the sequence of chloroplast genome were reported by various plant chloroplast genome studies (Doyle *et al.,* 1996; Tangphatsornruang *et al.,* 2011; Walker *et al.,* 2015; Sun *et al.,* 2017). These rearrangements occur as a result of contractions, expansions and inversions in the single copy regions (large single copy and small single copy) and the inverted repeats (Palmer *et al.,* 1987; Tangphatsornruang *et al.,* 2009). The rearrangements of the genes and inversion in the chloroplast genome are reported to be useful in phylogenetic analyses to solve taxonomic problems at various taxonomic levels because they do not occur often and estimation of their homology and inversion event polarity is simple (Johansson, 1999; Lee *et al.,* 2007; Jansen *et al.,* 2008; Yan *et al.,* 2017). With the importance of complete chloroplast genome in evolutionary studies, resolving phylogenetic relationship issues and the large number of genera and species in Acanthaceae, only complete chloroplast genome of 8 genera have been so far reported (*Andrographis paniculata* (Burm.f.) Nees, NC_022451; *Ruellia breedlovei* T. F. Daniel, KP300014; *Strobilanthes cusia* (Nees) O. Kuntze, MG874806 and four species *Echinacanthus* MF490441, MH045155, MH045156, and MH045157). Seven genera belong to cystolith clade, and only 1 genus belongs to non cystolith clade

In this study, we reported the characteristics of the complete chloroplast genome of *Blepharis ciliaris*, the second cp genome to be sequenced in Acantheae lineage. Moreover, its simple sequence repeats were reported to provide the tools for genetic diversity and identification of the species and lastly to resolve the status of Acantheae

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2. Materials and Methods

2.1. Plant material and DNA extraction

Plant material (vegetative and floral part) was collected through field survey of *B. ciliaris* in North Jeddah, Saudi Arabia. Plant identification and DNA extraction were done according to Samaila *et al*., (2019).

2.2. Library construction, sequencing and assembly

A total amount of 1.0µg DNA was used as input material for the DNA sample preparations. Sequencing libraries were generated using NEBNext® DNA Library Prep Kit following the manufacturer's recommendations and indices were added to each sample (Samaila *et al.*, 2019). The raw reads were filtered to get the clean reads (5 Gb) using PRINSEQ lite v0.20.4 (Schmieder and Edwards, 2011) and were subjected to de novo assembly using NOVOPlasty2.7.2 (Dierckxsens *et al*., 2016) with kmer K-mer= 31 (Samaila *et al.*, 2019)

2.3. Gene annotation

The program DOGMA (Dual Organellar Genome Annotator, University of Texas at Austin, Austin, TX, USA) (Wyman, *et al*., 2004) was used to annotate the genes in the assembled chloroplast genome. The positions of start and stop codon were adjusted manually. trnAscan-SE server (http://lowelab.ucsc.edu/tRNAscan-SE/)(Schattner et al., 2005) was used to verify the tRNA genes and finally, the plastome genome circular map was drawn using OGDRAW (Organellar Genome DRAW) (Lohse *et al.*, 2007). The sequence of the chloroplast genome of *B. ciliaris* was deposited in the GenBank database with the accession number (MK548576).

2.4. Sequence Analysis

MEGA 6.0 was used to analyze the relative synonymous codon usage values (RSCU), base composition and codon usage. Possible RNA editing sites present in the protein coding genes of *B. ciliaris* cp genome were determined using PREP suite (Kurtz et al., 2001) with 0.8 as the cutoff value.

2.5. Repeat analysis in B. ciliaris chloroplast genome

Simple sequence repeats (SSRs) were identified in the *B. ciliaris* chloroplast genome using the online software MIcroSAtellite (MISA) (Thiel et al., 2003) with the following parameters: eight, five, four and three repeats units for mononucleotides, dinucleotides, trinucleotides and tetra, penta, hexa nucleotides SSR motifs respectively. For analysis of long repeats (palindromic, forward, reverse and complement) the program REPuter (https://bibiserv.cebitec.uni-bielefeld.de/reputer) (Kurtz et al., 2001) with default parameters was used to identify the size and location of the repeats in *B. ciliaris* chloroplast genome

3. Results and Discussion

3.1. Characteristics of B.ciliaris chloroplast genome

The complete plastome sequence of *B. ciliaris* has a circular and quadripartite structure. The total length of the genome is 149, 717 bp (Samaila et al., 2019). The plastome has four distinct regions which are Small Single Copy (SSC), Large Single Copy (LSC), and a pair of Inverted repeats (IRa and IRb) which separate the small single copy and large single copy (Figure 1). The region coding for genes is 87, 073bp in length which constitutes 58.15% of the genome, the remaining 62, 644 bp is the non-coding region which includes intron and intergenic spacer (41.85%). The length of the SSC, LCS, IRa and IRb is 16, 998 bp, 82, 057 bp, 25, 331 bp and 25, 331 bp, respectively. The LSC and SSC regions possessed GC content of 36.6 % and 32.4 %, respectively, while the inverted repeats IRa and IRb have 43.6 % (Table 1). The architecture of the *B. ciliaris* cp genome is like other reported Acanthoideae cp genomes (Chunming et al., 2019; Yongbin and Erin, 2017). The plastome sequence has GC of 38.5% and AT content of 61.5%; this is consistent with the plastome data of *Strobilanthes cusia* (Chen et al., 2018). The percentage of GC in the inverted repeat regions is found to be higher than the large single copy region and small single region.

Table 1. Base composition in the *B. ciliaris* chloroplast genome.

Region		T(U)	C	A	G	Total
cp genome		31.1	19.6	30.4	18.9	149717.0
LSC		32.4	18.8	31.0	17.8	82057.0
SSC		33.7	16.7	33.9	15.7	16998.0
IRA		28.3	22.6	28.1	21.0	25331.0
IRB		28.1	21.0	28.3	22.6	25331.0
	1st Position	31	19.5	30.4	18.7	49906.0
	2nd Position	31	19.6	30.0	19.1	49906.0
	3rd Postion	31	19.7	30.7	19.0	49905.0

The complete chloroplast genome of *B. ciliaris* contained a total of 131 genes; 113 genes out of the 131 are unique and are present in the single copy regions (large single copy containing 83 genes and the small single copy contained 13 genes); 18 genes are duplicated in the pair of the inverted repeat region which include 8 protein-coding genes, 4 rRNAs and 7 tRNAs. There are 79 protein-coding genes, 4 rRNAs and 30 tRNAS in the plastome (Table 2 and Figure 1). The structural organization and the number of genes in the plastome is consistent with that of the sequenced Acanthoideae cp genome (Chunming et al., 2019; Yongbin and Erin, 2017). Irregular start codons like ACG, GTG and ATC were discovered in some of the annotated genes while majority of genes starts with normal start codon ATG. This phenomenon is also present in some genes in the sequenced chloroplast genome of angiosperms (Chen et al., 2018; Park et al., 2017; Li et al., 2017).

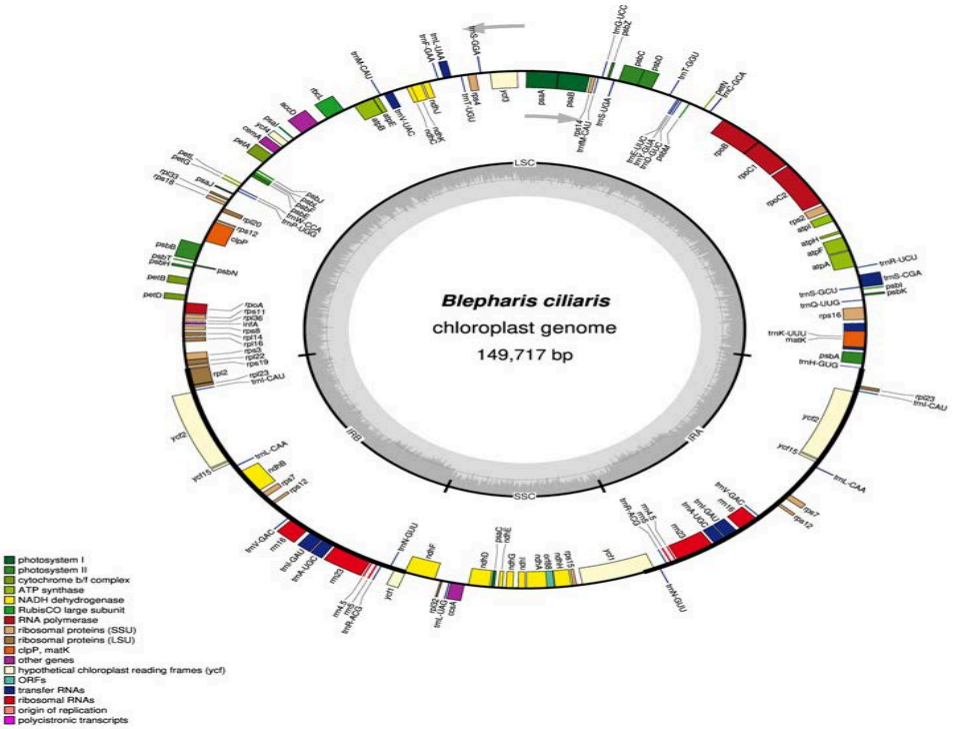

Figure 1. Gene map of the *B. ciliaris* chloroplast genome. Genes outside the circles are transcribed in counter-clockwise direction and those inside in clockwise direction. Known functional genes are indicated in the colored bar. The GC and AT contents are denoted by the dark grey and light grey color in the inner circle respectively. LSC indicates large single copy; SSC indicates small single copy, and IR indicates inverted repeat.

Table 2. Genes present in the chloroplast genome of *B. ciliaris*

Category	Group of genes	Name of genes
RNA genes	ribosomal RNA genes (rRNA)	*rrn5*, *rrn4.5*, *rrn16*, *rrn23*
	Transfer RNA genes (tRNA)	*trnH-GUG*, *trnK-UUU*$^{+}$, *trnQ-UUG*, *trnS-GCU*, *trnS-CGA*$^{+}$, *trnR-UCU*,*trnC-GCA*, *trnD-GUC*, *trnY-GUA*, *trnE-UUC*, *trnT-GGU*, *trnS-UGA*, *trnfM-CAU*, *trnG-GCC*, *trnS-GGA*, *trnL-UAA*$^{+}$, *trnT-UGU*, *trnF-GAA*, *trnV-UAC*$^{+}$, *trnM-CAU*, *trnW-CCA*, *trnP-UGG*, *trnI-CAU*^{a}, *trnL-CAA*^{a}, *trnV-GAC*^{a}, *trnI-GAU*$^{+,a}$, *trnA-UGC*$^{+,a}$, *trnR-ACG*^{a}, *trnN-GUU*^{a}, *trnL-UAG*,
Ribosomal proteins	Small subunit of ribosome	*rps2*, *rps3*, *rps4*, *rps7*^{a}, *rps8*, *rps11*, *rps12*^{a}, *rps14*, *rps15*, *rps*,*16*$^{+}$, *rps18*,*rps19*
Transcription	Large subunit of ribosome	*rpl2*$^{+,a}$, *rpl14*, *rpl16*, *rpl20*, *rpl22*, *rpl23*^{a}, *rpl32*, *rpl33*, *rpl36*
	DNA dependent RNA polymerase	*rpoA*, *rpoB*, *rpoC1*$^{+}$, *rpoC2*
Protein genes	Photosystem I	*psaA*, *psaB*, *psaC*, *psaI*,*psaJ*,*ycf3*$^{++}$
	Photosystem II	*psbA*, *psbB*, *psbC*, *psbD*, *psbE*, *psbF*, *psbH*, *psbI*, *psbJ*, *psbK*, *psbL*, *psbM*, *psbN*, *psbT*, *psbZ*
	Subunit of cytochrome	*petA*, *petB*, *petD*, *petG*, *petL*, *petN*
	Subunit of synthase	*atpA*, *atpB*, *atpE*, *atpF*$^{+}$, *atpH*, *atpI*
	Large subunit of rubisco	*rbcL*
	NADH dehydrogenase	*ndhA*$^{+}$, *ndhB*$^{+a}$, *ndhC*, *ndhD*, *ndhE*, *ndhF*, *ndhG*, *ndhH*, *ndhI*, *ndhJ*, *ndhK*
	ATP dependent protease subunit P	*clpP*$^{++}$
	Chloroplast envelope membrane protein	*cemA*
Other genes	Maturase	*matK*
	Subunit acetyl-coA carboxylase	*accD*
	C-type cytochrome synthesis	*ccsA*
	Hypothetical proteins	*ycf2*^{a},*ycf4*, *ycf15*^{a}
	Component of TIC complex	*ycf1*^{a}

$^{+}$ Gene with one intron, $^{++}$ Gene with two intron and ^{a} Gene with copies

Some of the annotated genes in the chloroplast genome of *B. ciliaris* contained intron, like the sequenced cp genomes of Lamiales (Li *et al.*, 2017, Josphat *et al.*, 2018). Among the 113 coding genes, 14 contained the intron (Table 3). Out of the 14 genes with intron, 8 are protein coding genes and 6 are tRNAs. The large single copy region contained 12 genes while the other four are situated in the inverted repeat region which includes *ndhB, trnA-UGC,trnI-GAU* and *rpl2*. Two genes, *clpP* and *ycf3* have 2 introns and the other 12 genes have only 1 intron; this has been reported in cp genome of *S. cusia* (Chen *et al.*, 2018). *trnK-UUU* has the longest intron while *trnL-UAA* has the shortest (Table 3).

Table 3: Genes with introns in the *B. ciliaris* chloroplast genome and length of introns and exons

Gene	Location	Exon I (bp)	Intron I (bp)	Exon II (bp)	Intron II (bp)	Exon III (bp)
rps16	LSC	34	902	225		
atp F	LSC	143	658	470		
rpoC1	LSC	434	783	1628		
ycf3	LSC	128	716	227	716	152
clpP	LSC	68	731	290	557	275
rpl2	IR	392	669	434		
ndhB	IR	776	685	755		
ndhA	SSC	551	905	539		
trnK-UU	LSC	36	2458	37		
trnS-CGA	LSC	57	1059	37		
trnL-UAA	LSC	36	489	49		
trnV-UAC	LSC	37	578	36		
trnI-GAU	IR	41	943	34		
trnA-UGC	IR	37	810	34		

The frequency of the codon usage present in the chloroplast genome was computed using the nucleotide sequence of protein-coding genes and tRNA genes 87, 073bp; the relative synonymous codon usage of the genes in the genome is presented in (Table 4). The results showed the genes in the plastome are encoded by 25, 233 codons. Codons that code for the amino acids leucine appear more frequently in the genome 2, 762 (11.0%) (Figure 2), like that of *Justicia flava* (Samaila et al., 2019), whereas codons coding for Trytophan are the least 446 (1.90%) in the genome. Guanine and Cytosine ending are found to be more frequent than their counterpart Adenine and Thymine; this is not the case in other plastome sequences (Zhou *et al.*, 2017; Jiang *et al.*, 2017; Zhou *et al.*, 2018). The result of the analysis (Table 4) showed that codon usage bias is low in the chloroplast genome of *B. ciliaris*. The RSCU values of 30 codons were greater than1 and all of them have A/T ending while for 31 codons were less than 1 and are all of G/C ending. Only two amino acids Tryptophan and Methionine have RSCU value of 1; therefore, they are the only amino acids with no codon bias.

The program PREP suite was used to predict the RNA editing site in the chloroplast genomes of *B. cilirias.* The first codon of the first nucleotide was used in all the analysis. The result of the analysis shows that most of the conversions in the codon positions are from the amino acid serine to leucine (Table 5). In all, the program revealed 50 editing sites in the genome and they are distributed within 20 protein-coding genes. The gene *ndhB* has the highest number of editing sites with (9 sites); this is consisted with previous researches (Wang *et al.*, 2017; Kumbhar *et al.*, 2018; Park *et al.*, 2018). Other genes with highest number of editing sites in the genome are *rpoB* and *rpoC2* having 6 and 5 editing sites, respectively. The following genes: *accD, atpF, atpI, ndhG, rpl2, rpl20, rpoA* and *rps2* have the lowest number of editing site with 1 editing site. Conversions of proline to serine were observed, which involves the changing of the amino acids in the RNA editing site from apolar to polar group. Genes such as *atpB, ccsA, clpP, ndhC, ndhE, ndhG, petD, petG* and *petL*, among others, do not possess RNA predicting site in their first codon of the nucleotide.

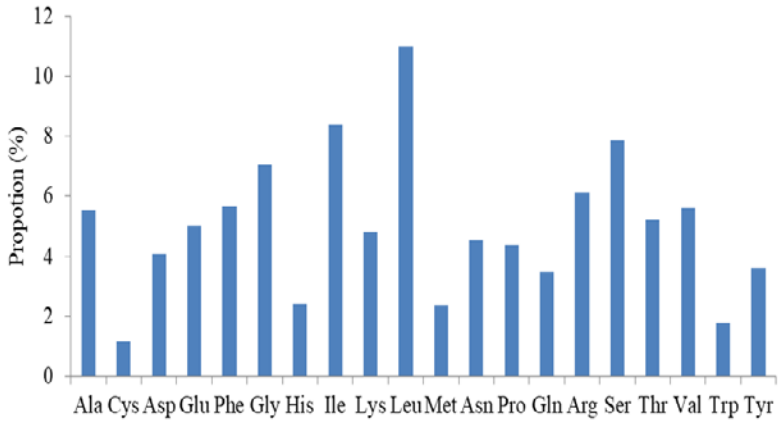

Figure 2: Amino acids frequencies in *B. ciliaris* chloroplast genome protein coding sequences

Table 4: Codon – anticodon recognition patterns and codon usage of the *B. ciliaris* chloroplast genome

Codon	Amino Acid	RSCU	tRNA	Codon	Amino Acid	RSCU	tRNA
UUU	Phe	1.29	*trnF-GAA*	UAU	Tyr	1.61	*trnY-GUA*
UUC	Phe	0.71		UAC	Tyr	0.39	
UUA	Leu	1.82	*trnL-UAA*	UAA	Stop	1.55	
UUG	Leu	1.23	*trnL-CAA*	UAG	Stop	0.74	
CUU	Leu	1.26	*trnL-UAG*	CAU	His	1.57	*trnH-GUG*
CUC	Leu	0.43		CAC	His	0.43	
CUA	Leu	0.86		CAA	Gln	1.53	*trnQ-UUG*
CUG	Leu	0.39		CAG	Gln	0.47	
AUU	Ile	1.48	*trnI-GAU*	AAU	Asn	1.51	*trnG-GUU*
AUC	Ile	0.67		AAC	Asn	0.49	
AUA	Ile	0.85	*trnI-CAU*	AAA	Lys	1.43	*trnK-UUU*
AUG	Met	1	*trnM-CAU*	AAG	Lys	0.57	
GUU	Val	1.49	*trnV-GAC*	GAU	Asp	1.62	*trnD-GUC*
GUC	Val	0.52		GAC	Asp	0.38	
GUG	Val	1.43		GAA	Glu	1.45	*trnE-UUC*
GUA	Val	0.56	*trnV-UAC*	GAG	Glu	0.55	
UCU	Ser	1.72	*trnS-GGA*	UGU	Cys	1.53	*trnC-GCA*
UCC	Ser	0.98		UGC	Cys	0.47	
UCG	Ser	1.17		UGA	Stop	0.71	
UCA	Ser	0.61	*trnS-UGA*	UGG	Trp	1	*trnW-CCA*
CCU	Pro	1.45	*trnP-UGG*	CGU	Arg	1.25	*trnR-ACG*
CCC	Pro	0.83		CGC	Arg	0.43	*trnR-UCU*
CCA	Pro	1.11		CGA	Arg	1.35	
CCG	Pro	0.61		CGG	Arg	0.51	
ACU	Thr	1.61		AGA	Arg	1.17	
ACC	Thr	0.76		AGG	Arg	0.35	
ACG	Thr	1.16	*trnT-GGU*	AGU	Ser	1.75	*trnS-GCU*
ACA	Thr	0.47	*trnT-UGU*	AGC	Ser	0.71	
GCU	Ala	1.77	*trnA-UGC*	GGU	Gly	1.26	*trnG-GCC*
GCC	Ala	0.65		GGC	Gly	0.42	
GCA	Ala	1.13		GGA	Gly	1.61	
GCG	Ala	0.45		GGG	Gly	0.71	*trnG-UCC*

Table 5. Predicted RNA editing sites in the *B. ciliaris* chloroplast genome.

gene	Nucleotide Position	Amino Acid Position	Codon Conversion	Amino Acid Conversion	Score
accD	230	77	TCT => TTT	S =>F	1
atpA	791	264	CCC => CTC	P=> L	1
	914	305	TCA => TTA	S => L	1
atpF	92	31	CCA => CTA	P=> L	0.86
atpI	620	207	TCA => TTA	S=> L	1
ccsA	71	24	ACT => ATT	T => I	1
	377	126	ACC => ATC	T => I	0.86
matK	70	24	CTT => TTT	L => F	1
	620	207	TCT => TTT	S =>F	0.86
	640	214	CAT => TAT	H => Y	1
	1249	417	CAT => TAT	H => Y	1
ndhA	341	114	TCA => TTA	S => L	1
	875	292	TCT => TTT	S => F	1
	1073	358	TCC => TTC	S => F	1

ndhB	149	50	TCA => TTA	S => L	1
	467	156	TCA => TTA	S => L	1
	586	196	CAT => TAT	H => Y	1
	737	246	CCA => CTA	P=> L	1
	746	249	TCT => TTT	S => F	1
	830	277	TCA => TTA	S => L	1
	836	279	TCA => TTA	S => L	1
	1292	431	TCC => TTC	S => F	1
	1481	494	CCA => CTA	P => L	1
ndhD	20	7	ACG => ATG	T => M	1
	563	188	GCT => GTT	A => V	1
	896	299	TCA => TTA	S => L	1
ndhF	671	224	CCA => CTA	P => L	1
	1535	512	ACC => ATC	T => I	1
ndhG	314	105	ACA => ATA	T => I	0.8
petB	424	142	CGG => TGG	R => W	1
	617	206	CCA => CTA	P => L	1
rpl2	596	199	GCG => GTG	A => V	0.86
rpl20	308	103	TCA => TTA	S => L	0.86
rpoA	887	296	TCG => TTG	S => L	1
rpoB	338	113	TCT => TTT	S => F	1
	473	158	TCA => TTA	S => L	0.86
	551	184	TCA => TTA	S => L	1
	566	189	TCG => TTG	S => L	1
	2000	667	TCT => TTT	S => F	1
	2426	809	TCA => TTA	S => L	0.86
rpoC2	1726	576	CTC => TTC	L => F	0.86
	1909	637	CCA => TCA	P => S	1
	2248	750	CCC => TCC	P => S	1
	3127	1043	CGT => TGT	R => C	0.8
	3731	1244	TCA => TTA	S => L	0.86
rps2	248	83	TCA => TTA	S => L	1
rps14	80	27	TCA => TTA	S => L	1
	149	50	CCA => CTA	P => L	1
ycf3	374	125	ACT => ATT	T => I	1
	388	130	CCT => TCT	P => S	0.86

3.2. Repeat Analysis

3.2.1. Long repeats

The program REPuter was used to identify long repeat sequences present *B. ciliaris* chloroplast genome using default settings; from the result it was discovered that all the four types of repeats (palindromic, forward, reverse and complement) were present in the plastome of *B. ciliaris* (Table 6). The analysis showed 22 palindromic repeats, 17 forward repeats, 7 reverse repeats and 3 complement repeats (Table 6). In total, there are 49 repeats in the chloroplast genome of *B. ciliaris*. The majority of the repeats size are between 20-29 bp (61.22%), followed by 10-19bp (28.57%), whereas 30-39 bp and 40-49 bp are the least with (6.12% and 4.08%), respectively. In the first location, the intergenic spacer harbored 65.30% of the repeats; this has also been reported in cp genome of *Fagopynon dibotrys* (Xumei *et al.*, 2018). The tRNA contained 4 repeats (8.16%) and 11 repeats (22.44%) are located in the protein coding genes. Within the protein coding genes only *rpoC2*, *ndhC*, *psaB*, *ycf2* and *ycf1* contained the longest repeats.

Table 6: Repeat sequences present in the *B. ciliaris* chloroplast genome.

S/N	Repeat Size	Repeat Position 1	Repeat Type	Repeat Location 1	Repeat Position 2	Repeat Location 2	E-Value
1	41	96141	F	IGS	116548	IGS	1.30E-15
2	41	116548	P	IGS	135532	IGS	1.30E-15
3	32	35433	P	IGS	35433	IGS	3.42E-10
4	30	7483	P	IGS-*trnS-GCU*	44096	IGS-*trnS-GGA*	5.47E-09
5	30	113424	P	IGS	113424	IGS	5.47E-09
6	26	85621	P	*ycf2*	85621	*ycf2*	1.40E-06
7	26	85621	F	*ycf2*	146067	*ycf2*	1.40E-06
8	26	146067	P	*ycf2*	146067	*ycf2*	1.40E-06
9	23	42629	F	*ycf3* Intron	96143	IGS	8.96E-05
10	23	42629	F	IGS	116550	*ndhA* Intron	8.96E-05
11	23	42629	P	IGS	135548	IGS	8.96E-05
12	23	59041	F	IGS	59062	IGS	8.96E-05
13	22	9018	F	*trnG-GCC*	35559	*trnG-UCC*	3.58E-04
14	22	9075	P	IGS	9127	IGS	3.58E-04
15	22	89166	F	*ycf2*	89184	*ycf2*	3.58E-04
16	22	89166	P	*ycf2*	142508	*ycf2*	3.58E-04
17	22	89184	P	*ycf2*	142526	*ycf2*	3.58E-04
18	22	91574	P	IGS	91600	IGS	3.58E-04
19	22	91574	F	IGS	140092	IGS	3.58E-04
20	22	91600	F	IGS	140118	IGS	3.58E-04
21	22	140092	P	IGS	140118	IGS	3.58E-04
22	22	142508	F	*ycf2*	142526	*ycf2*	3.58E-04
23	21	7489	F	*trnS-GCU*	34623	*trnS-UGA*	1.43E-03
24	21	34623	P	*trnS-UGA*	44099	*trnS-GGA*	1.43E-03
25	21	35438	R	IGS	35438	IGS	1.43E-03
26	21	35438	C	IGS	35439	IGS	1.43E-03
27	21	35439	R	IGS	35439	IGS	1.43E-03
28	21	35765	F	*trnfM-CAU*	64866	*trnP-UGG*	1.43E-03
29	21	111719	P	IGS	111759	IGS	1.43E-03
30	20	35438	P	IGS	35438	IGS	5.73E-03
31	20	35438	F	IGS	35440	IGS	5.73E-03
32	20	35440	P	IGS	35440	IGS	5.73E-03
33	20	49223	R	*ndhC*	49223	*ndhC*	5.73E-03
34	20	72300	P	IGS	72324	IGS	5.73E-03
35	20	121066	P	*ycf1*	121066	*ycf1*	5.73E-03
36	19	26706	F	IGS	36178	*rps14*	2.29E-02
37	19	35438	R	IGS	35438	IGS	2.29E-02
38	19	35438	C	IGS	35441	IGS	2.29E-02
39	19	35441	R	IGS	35441	IGS	2.29E-02
40	19	59093	R	IGS	59093	IGS	2.29E-02
41	19	68921	R	IGS	68921	IGS	2.29E-02
42	19	109803	F	IGS	109881	IGS	2.29E-02
43	18	7894	C	IGS	35739	IGS	9.17E-02
44	18	8131	P	IGS	8131	IGS	9.17E-02
45	18	15894	P	*rpoC2*	59092	IGS	9.17E-02
46	18	35438	P	IGS	35438	IGS	9.17E-02
47	18	35438	F	IGS	35442	IGS	9.17E-02
48	18	35442	P	IGS	35442	IGS	9.17E-02
49	18	37221	F	*psaB*	39436	*psaA*	9.17E-02

3.2.2. Simple sequence repeats (SSRs)

There are short repeats of nucleotide series (1-6 bp) that are dispensed all through genome called microsatellites (SSRs).These short repeats in plastid genome are passed from a single parent. As a result, they are used as molecular indicators in developmental studies such as genetic heterogeneity, and also contribute in recognition of species (Bryan *et al.*, 1999; Provan, 2000; Ebert and Peakall, 2009). A total of 91 microsatellites were found in the chloroplast genome of *B. ciliaris* in this study (Table 7). Majority of SSRs in the cp genome are mononucleotide (83.51%) of which the majority are poly T and A (Figure 4). Poly T (polythymine) constituted 40.65%, whereas poly A (polyadenine) 38.46%; this is consistent with previous studies (Saina *et al.*, 2018; Zhou *et al.*, 2016). Only two poly C (polycytosine) and poly G (polyguanine) were present in the genome, each with 2.17%. Among the dinucleotide only AT/AT is found in the genome. Reflecting series complementary, two trinucleotide AAG/CTT, and AAT/ATT, five tetraAAAT/ATTT, AATC/ATTG, AATT/AATT,

ACAG/CTGT were present in the cp genome. Penta and hexa nucleotides were not discovered in the genome (Figure 4). The intergenic spacer region harbored most of the microsatellite (68.13%) than the coding region (31.86%) (Figure 5). Most, but not all the repeats (70.40%) were detected in the LSC region, and the SSC region incorporates the least number of repeats in the genome.

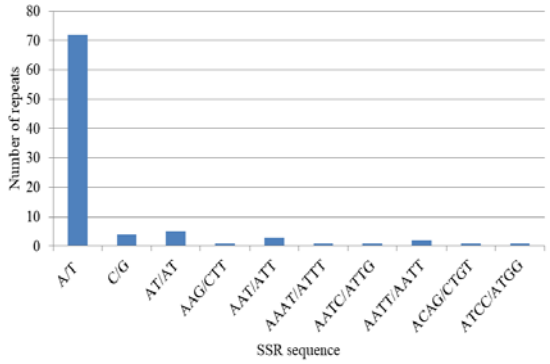

Figure 4: Frequency of different SSR motifs in different repeat types in *B. ciliaris* chloroplast genome

Table 7. Simple sequence repeats in the chloroplast genome of *B. ciliaris*

Repeat Motif	Length	start	End	Region	Annotation
(T)9	9	291	300	LSC	IGS
(AATT)3	3	5403	5414	LSC	IGS
(T)9	9	7230	7238	LSC	IGS
(T)8	8	7902	7909	LSC	IGS
(TA)5	5	7932	7941	LSC	IGS
(T)8	8	8404	8411	LSC	IGS
(A)8	8	9269	9276	LSC	IGS
(GTCT)3	3	10457	10468	LSC	IGS
(T)9	9	11716	11724	LSC	IGS
(T)9	9	12402	12410	LSC	IGS
(T)8	8	13743	13750	LSC	IGS
(T)9	9	14752	14760	LSC	IGS
(TA)5	5	14780	14789	LSC	IGS
(A)9	9	15600	15608	LSC	IGS
(T)9	9	15898	15906	LSC	*rpoC2*
(T)10	10	17802	17811	LSC	*rpoC2*
(A)8	8	17944	17951	LSC	*rpoC2*
(A)8	8	21615	21622	LSC	*rpoC1*
(T)8	8	25538	25545	LSC	*rpoB*
(A)8	8	30385	30392	LSC	IGS
(TTC)4	4	34253	34264	LSC	*psbC*
(T)11	11	34467	34477	LSC	IGS
(A)12	12	35239	35258	LSC	IGS
(A)9	9	35308	35316	LSC	IGS
(TA)11	11	35439	35460	LSC	IGS
(A)8	8	35747	35754	LSC	IGS
(T)8	8	42201	42208	LSC	IGS
(A)9	9	43289	43297	LSC	IGS
(A)8	8	43865	43872	LSC	IGS
(T)9	9	45059	45067	LSC	IGS
(A)9	9	46316	46324	LSC	IGS
(A)11	11	46494	46504	LSC	IGS
(T)8	8	46937	46944	LSC	IGS
(A)8	8	49698	49705	LSC	IGS
(TAT)4	4	51250	51261	LSC	IGS
(ATGG)4	4	51301	51316	LSC	IGS
(T)10	10	53435	53444	LSC	*AtpB*
Repeat Motif	Length	start	End	Region	Annotation
(ATA)4	4	53489	53500	LSC	IGS
(AAT)4	4	54070	54081	LSC	IGS
(G)8	8	57533	57540	LSC	*accD*
(ATCA)3	3	58090	58101	LSC	IGS
(TA)6	6	58156	58167	LSC	IGS
(A)9	9	59099	59107	LSC	IGS
(T)8	8	59334	59341	LSC	*ycf4*
(T)8	8	59773	59780	LSC	IGS
(A)8	8	61221	61228	LSC	*petA*
(A)8	8	62298	62305	LSC	IGS
(A)8	8	62907	62914	LSC	*psbF*
(A)8	8	66648	66655	LSC	IGS
(A)8	8	67389	67396	LSC	IGS
(A)8	8	68737	68744	LSC	IGS
(A)9	9	68866	68874	LSC	IGS
(A)12	12	69359	69370	LSC	IGS
(T)8	8	71197	71204	LSC	*PsbB*
(T)8	8	72022	72029	LSC	IGS
(A)10	10	72115	72124	LSC	IGS
(T)8	8	73151	73158	LSC	IGS
(T)10	10	76123	76132	LSC	*rpoA*
(T)8	8	78010	78017	LSC	IGS
(T)8	8	81973	81980	LSC	*rps19*
(T)9	9	82005	82013	LSC	*rps19*
(A)9	9	87411	87419	IRb	*ycf2*
(A)8	8	90040	90047	IRb	*ycf2*
(A)9	9	94536	94544	IRb	IGS
(T)8	8	96519	96526	IRb	IGS
(T)8	8	100252	100259	IRb	IGS
(G)8	8	101469	101476	IRb	IGS
(A)9	9	107656	107664	SSC	*ndhF*
(A)8	8	108920	108927	SSC	*ndhF*
(AATA)3	3	112096	112107	SSC	*ndhD*
(T)8	8	112487	112494	SSC	*ndhD*
(A)8	8	113461	113468	SSC	IGS
(A)8	8	116785	116792	SSC	IGS
(T)9	9	119309	119317	SSC	*rps15*
(A)8	8	119521	119528	SSC	*rps15*
Repeat Motif	Length	start	End	Region	Annotation
(T)9	9	121498	121506	SSC	*ycf1*
(AATT)3	3	121516	121527	SSC	*ycf1*
(T)8	8	121529	121536	SSC	*ycf1*
(T)11	11	121956	121966	SSC	*ycf1*
(T)8	8	121979	121986	SSC	*ycf1*
(A)9	9	122030	122038	SSC	*ycf1*
(T)8	8	122497	122504	SSC	*ycf1*
(T)9	9	122563	122571	SSC	*ycf1*
(A)8	8	123209	123216	SSC	*ycf1*
(C)8	8	130239	130246	IRa	IGS
(A)8	8	131456	131463	IRa	IGS
(A)8	8	135189	135196	IRa	IGS
(T)9	9	137171	137179	IRa	IGS
(T)8	8	141668	141675	IRa	*ycf2*
(T)9	9	144296	144304	IRa	*ycf2*

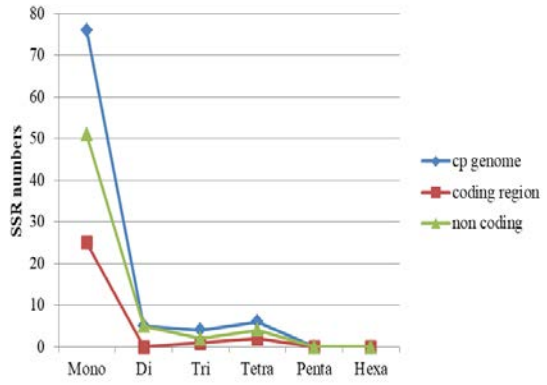

Figure 5: Number of SSR types in complete genome, protein coding regions and Non coding genes.

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Effect of *Ficus exasperata* VAHL Extracts on Bacterial Isolates Associated with HIV Infection

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Abstract

Opportunistic infections are mostly associated with HIV infected patients owing to their immune-compromised situation. This study is aimed at investigating the antibacterial effects of *Ficus exasperata* leaf extracts on bacterial isolates from blood samples of HIV infected patients attending antiretroviral therapy (ART) clinics in Akure, Nigeria. A total of 233 blood samples were each collected and subjected to bacteriological analyses. Phytochemical constituents of the leaf extracts were done using standard methods. Extracts were further purified using column chromatography. Antibiotics sensitivity test and antibacterial activity of *F. exasperata* extracts on the isolated bacteria was done using disc and agar well diffusion test respectively. Plasmid profile of multiple resistant bacterial isolates was also investigated. Ciprofloxacin exhibited the highest efficacy across all the isolates compared to other antibiotics. Methanol extract at 400 mg/ml compared favorably well with ciprofloxacin and exhibited the highest antibacterial efficacy on *E. coli* (22.13 ± 0.25^{h}) while chloroform extract showed the least. Purified extracts recorded higher efficacy compared to the crude extracts. The highest activity was recorded for methanol extract at 200 mg/ml against *K. pneumoniae* (32.98 ± 0.82^{d}) while the least was aqueous extract against *E. coli* (17.96 ± 0.33^{a}). The isolates showed multiple bands containing plasmids; however, most of the resistance was not plasmid based. This antibacterial efficacy of *F. exasperata* leaf extracts justifies its ethnomedicinal use as an alternative or complementary drug for the treatment of secondary bacterial infections commonly associated with HIV infection.

Keywords: Antibacterial, HIV, Blood, Plasmid, Antibiotics, Resistance, Ethnomedicine, Infections

1. Introduction

Human immunodeficiency virus and acquired immunodeficiency syndrome is a global pandemic (Cohen *et al.*, 2008). As of 2017, approximately 37 million people have HIV infection worldwide with the number of new cases that year being about 2 million. Of these 37 million, more than half are women and 2.6 million are less than 15 years old. It resulted in about 1.2 million deaths in 2014, down from a peak of 2.2 million in 2005 (UNAIDS, 2018). The clinical manifestation of HIV secondary infections in developing countries, including Nigeria, shows a high prevalence of infections of the skin, gut, respiratory tract, tuberculosis, and malnutrition (Akinsete *et al.*, 1998). Bacterial bloodstream infections constitute a significant public-health problem and present an important cause of morbidity and mortality in HIV-infected patients (Adeleye *et al*., 2010). Plants have continued to be a major resource for therapeutic purposes. Ethnobotanical and ubiquitous plants serve as a rich resource of natural drugs for research and development (Kong *etal.,* 1999). The natural composition of medicinal plants may act as new alternative in treating various emerging infectious diseases (Wurocheke *et al*., 2008; Ncube *et al*., 2008; Oluduro and Aderiye, 2009). Herbal medicine as a form of complementary and alternative medicine in the treatment of diseases is becoming increasingly popular in both developing and developed countries (Egwaikhide and Gimba, 2007; Mustapha *et al*., 2009). Phytomedicine has demonstrated its contribution to the reduction of excessive mortality, morbidity and disability due to diseases such as HIV/AIDS, malaria, tuberculosis, sickle cell anaemia, diabetes, mental disorders (Elujoba *et al*, 2005) and microbial infections (Iwu *et al.*, 1999). *Ficus exasperata* belongs to the family Moraceae, with 800 species occurring in the warmer part of the world (Odunbaku *et al*., 2008). Nigeria are replete with over 45 different species of *Ficus* (Keay and Onochie, 1964), such as *F. glomosa, F. lecardi, F. goliath, F. capensis, F. ingens* and *F. elastica,* which can be found in the Savannah, rainforest, besides rivers and streams. *Ficus exasperata* is commonly known as sand paper tree ("Ewe ipin" in Yoruba) and is widely spread in West Africa in all kinds of vegetation and particularly in secondary forest re-growth. The leaf extract from *F. exasperata* has been reported to have diverse uses such as treating hypertensive patients (Buniyamin *et al*., 2007), heamostative opthalmia, coughs and haemorrhoid (Odunbaku *et al.*, 2008). In Nigeria, young leaves of *F. exasperata* are prescribed as a common anti-ulcer remedy. Various pharmacological actions such as anti-diabetic, lipid lowering andantifungal activities have been reported for *F. exasperata* (Sonibare*et al.,* 2006). Other industrial uses are for polishing woods (Cousins and Michael, 2002), stabilization of vegetable oils, suppression of foaming, supplement as food stock and antimicrobial (Odunbaku *et al.,* 2008). The activities of leaf extract of *F. exasperata* against some pathogenic

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organisms have been extensively investigated (Buniyamin *et al.*, 2007; Odunbaku *et al*., 2008). However, recent toxicity studies in rats involving crude aqueous and ethanol extract of the leaves have indicated potential hepatic and renal toxicity as reflected by significantly increased serum transaminases and bilirubin (Irenell and Chukwunonso, 2006). Among different parts of *F. exasperata*, leaves have received much attention from the researchers across the world and have been widely studied for various pharmacological activities such as antidiabetic, hypotensive, antioxidant, anti-inflammatory, antiarthritic, antinociceptive, anticonvulsant, anxiolytic, antiulcer, antipyretic, uterotonic and antimicrobial activities (Faiyaz *et al*., 2012). Antibiotics can literally save lives and are effective in treating illnesses; however, they have the potential to cause unwanted side effects. Medicinal plant products when compared to their synthetic counterparts minimize these adverse side effects (Gislence *et al.*, 2000). Bacterial blood stream infections caused by multi-drug resistant bacteria are the major cause of morbidity and in chronic stage mortality in patients with human immunodeficiency virus. Thus, this study aimed at evaluating the *in-vitro* antimicrobial effects of the methanol, aqueous and chloroform extract of the leave of *F.exasperata* on bacterial isolates from the blood samples of human immunodeficiency virus infected patients.

2. Materials and Methods

2.1. Ethical approval

Ethical approval was obtained from the Health Research Ethics Committee (HREC) of State Specialist Hospital, Akure, Nigeria. Patient consent was sought before the collection of samples.

2.2. Collection of blood samples and plant materials

In total, 233 blood samples from serologically confirmed HIV-1 infected patients attending the HIV Clinic State Specialist Hospital, Akure, Ondo State, Nigeria, were collected in EDTA bottles and immediately transported to the Laboratory for microbiological analyses. Leaves of *F. exasperata* were harvested and authenticated at the Department of Crop, Soil and Pest Management, Federal University of Technology, Akure, Nigeria.

2.3. Bacteriological analyses

Culture media including brain heart infusion broth, MacConkey agar, blood agar, chocolate agar, nutrient agar, Mueller Hinton agar, and nutrient broth were prepared according to manufacturer's specification. The blood samples were each cultured on the media and further identified using cultural and biochemical methods according to the methods of Cheesbrough (2010).

2.4. Preparation of leaf extracts of F. exasperata

Leaves of *F. exasperata* were washed to remove particles and foreign materials, left to air dry at room temperature on a clean surface until a constant weight of the sample was observed and thereafter ground into fine powder using electronic blender. The dried powdered leaves were stored at room temperature (Shahidi-Bonjar, 2004). Exactly 200 g of *F. exasperata* was measured into a container and each soaked with methanol, chloroform and water. The mixture was allowed to stand for 72 hours with intermittent stirring. This was followed by repeated filtration using sterile muslin cloth, non-absorbent cotton wool and Whatman filter paper. The filtrates were concentrated *in vacuo* at 40 ℃ using a rotary evaporator. The percentage yield of each extract was determined by comparing the weight of the yield and the initial weight of the powder extracted. The extracts obtained were preserved at 4 ℃ before use (Atata *et al.*, 2003).

2.5. Phytochemical analysis

Screening and identification of bioactive chemical constituents like alkaloids, glycosides, saponins, phenolic compounds, flavonoids, and tannins, in the medicinal plants under study were carried out using standard methods described by Trease and Evans (2002); Usman and Osuji (2007).

2.6. Antibiotics sensitivity test of bacterial isolates

Antibiotic sensitivity testusing commercially available antibiotics was performed on the bacterial isolates cultured on Mueller Hinton gar plates using standardized agar-discs diffusion technique as described by Clinical Laboratory Standard Institute (CLSI, 2016).

2.7. Antibacterial activity of F. exasperata leaf extract on bacterial isolates

Agar well diffusion technique was used to determine the *in-vitro* antibacterial activity of the crude extract. A 1 ml of 18 hours broth culture of each of the test bacterial suspension that have been adjusted to turbidity equivalent of 0.5 McFarland standard was pour plated on sterile Mueller-Hinton agar plates, 6 mm wells were bored and filled with 0.5 ml of each extracts. Dimethylsulfoxide (DMSO) and ciprofloxacin (500 µg) were used as the negative and positive control respectively.

2.8. Plasmid profile analyses of multi drug resistant bacterial isolates

Plasmid extraction and curing was carried out using methods described by Akinjogunla and Enabulele, (2010) on selected multiple drug resistant bacterial isolates.

2.9. Purification of extract using column chromatography

Purification of the extract was carried out using column chromatography as described by Atta *et al*. (2009) and Usha *et al.* (2010) using petroleum ether, chloroform and methanol in ratio 3:1:1 v/v as eluting solvent. The column was packed with silica gel (60-120 mesh). The fractions obtained were reconstituted with 30% DMSO and spotted on TLC plates. Fractions with the same retention factor (R_f) were pooled together.

2.10. Statistical Analysis

Data were presented as mean ± standard error (SE). The significance of difference between treatment groups was tested using two-way analysis of variance (ANOVA) and treatment means were compared with Duncan's New Multiple Range test using SPSS version 20 software, at $p < 0.05$ level of significance.

3. Results

3.1. Percentage occurrence of bacteria isolated from HIV infected blood

The occurrence of bacterial isolates from HIV infected blood samples is expressed as percentage as shown in Table 1. Nine bacteria including *Staphylococcus aureus, Streptococcus pneumoniae, S. pyogenes, Shigella* sp, *Escherichia coli*, *Klebsiella pneumoniae*, *Proteus mirabilis*, *Pseudomonas aeruginosa*, and *Salmonella typhi*were isolated. *Pseudomonas aeruginosa* had the highest occurrence (18%) while *P. mirabilis* had the least (6%).

Table 1. Percentage occurrence of bacteria isolated from HIV infected blood

Isolates	Frequency	Occurrence (%)
Pseudomonas aeruginosa	31	18
Klebsiella pneumoniae	28	16
Escherichia coli	24	14
Shigella sp	20	11
Streptococcus pneumoniae	19	11
Staphylococcus aureus	17	10
Salmonella typhi	15	09
Streptococcus pyogenes	12	07
Proteus mirabilis	10	06

3.2. Qualitative phytochemical constituents of F. exasperata leaf

Figure 1 shows the different phytochemicals inherent in the different extracts of *F. exasperata* leaf. It revealed the presence of flavonoids, tannins, terpenoids, steroidal and cardiac glycosides; alkaloids and saponins were found to be present. Alkaloid and flavonoids of methanolic leaf extract had the highest quantity of phytochemicals.

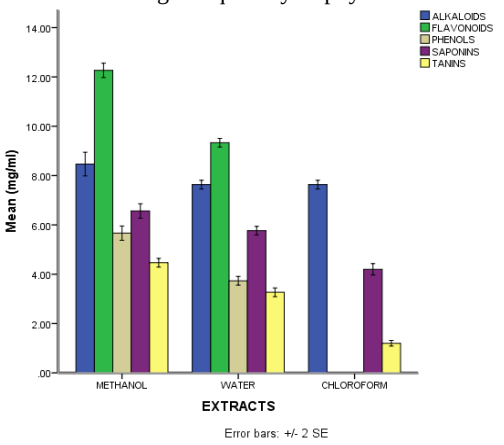

Figure 1. Quantitative Phytochemical Constituents of *F. exasperata* Leaf extract

3.3. Antibiotics sensitivity pattern of bacteria isolated from blood of HIV infected patients

Table 2 shows the antibiotics sensitivity patterns of Gram positive and negative bacteria isolated from the blood of HIV infected patients respectively. All the isolates showed multiple resistance to more than 3 antibiotics. Ciprofloxacin exhibited the highest efficacy across all the isolates compared to the other antibiotics.

Table 2. Antibiotics sensitivity pattern of bacteria isolated from HIV infected blood

Isolates	Zones of inhibition (diameter mm)									
Gram Positive	**ERY**	**OFL**	**STR**	**CHL**	**CEF**	**GEN**	**PEF**	**COT**	**CPX**	**AMX**
S. pyogenes	8.67±0.33^{f}	11.67±0.33^{a}	0.00±0.00^{e}	11.00±0.58^{b}	13.00±0.58^{b}	8.33±0.67^{a}	8.67±0.33^{d}	10.33±0.67^{a}	11.67±0.67^{a}	6.67±0.33^{b}
S. pneumoniae	13.33±0.33^{d}	19.00±0.58^{c}	17.67±0.89^{c}	24.33±0.33^{a}	17.00±0.58^{a}	17.00±0.58^{a}	19.67±0.33^{b}	15.00±0.80^{a}	15.00±0.58^{f}	20.67±0.67^{e}
S. aureus	9.00±0.58^{a}	0.00±0.00^{c}	10.33±0.58^{b}	12.33±0.58^{c}	11.00±0.67^{b}	1.67±0.33^{b}	12.00±0.58^{a}	9.67±0.33^{b}	10.00±0.58^{a}	11.67±0.67^{c}
Gram Negative	**AUG**	**TET**	**CEF**	**NIT**	**PEF**	**GEN**	**COT**	**OFL**	**AMX**	**CPX**
P. mirabilis	6.33±0.33^{d}	4.67±0.67^{c}	4.33±0.33^{a}	2.33±0.33^{a}	6.33±0.33^{c}	10.67±0.33^{a}	6.67±0.33^{b}	10.00±0.58^{d}	3.00±0.58^{a}	14.67±0.33^{a}
S. typhi	11.33±0.67^{b}	9.00±0.58^{c}	9.67±0.33^{a}	12.33±0.67^{a}	15.0±0.58^{d}	19.67±0.33^{a}	8.67±0.67^{f}	15.67±0.33^{a}	16.00±0.00^{b}	20.00±0.00^{e}
Shigella sp	9.33±0.33^{b}	10.67±0.33^{d}	22.67±0.33^{f}	16.33±0.3^{a}	15.67±0.30^{a}	17.67±0.33^{d}	20.00±0.58^{b}	19.00±0.58^{e}	15.00±0.58^{d}	20.67±0.33^{d}
E. coli	6.67±0.67^{e}	9.00±0.58^{g}	21.00±0.58^{b}	16.33±0.3^{a}	9.33±0.33^{f}	21.67±0.89^{e}	18.67±0.67^{f}	6.67±0.67^{b}	12.67±0.33^{a}	22.33±0.33^{f}
K. pneumoniae	8.33±0.33^{e}	0.00±0.00^{a}	6.00±0.00^{a}	6.67±0.67^{c}	4.67±0.33^{a}	7.00±0.58^{e}	0.33±0.33^{d}	0.00±0.00^{a}	0.00±0.00^{a}	15.33±0.33^{c}
P. aeruginosa	9.00±0.58^{a}	6.33±0.33^{a}	17.33±0.33^{h}	7.00±0.58^{b}	21.00±0.00^{f}	16.33±0.33^{d}	7.00±0.58^{f}	17.33±0.33^{a}	13.33±0.33^{b}	19.67±0.67^{d}

Data are presented as Mean ± S.E (n=3). Values with the same superscript letter(s) along the same column are not significantly different (P<0.05)
Keys: cotrimoxazole (25 µg) (COT), ciprofloxacin (CPX) (10 µg), tetracycline (30 µg) (TET), amoxicillin (30 µg) (AMX), ofloxacin (5 µg) (OFL), augmentin (30 µg) (AUG), nitrofurantoin (200 µg) (NIT), gentamycin (10 µg) (GEN), ceftriaxone (30 µg) (CEF), pefloxacin (5 µg) (PEF), streptomycin (10 µg) (STR), chloramphenicol (30 µg) (CHL) and erythromycin (15 µg) (ERY)

3.4. Antibacterial activity of F. exasperata leaf extracts on bacteria isolated from blood of HIV infected patients

Table 3 shows the antibacterial activity of varying concentrations of leaf extracts of *F. exasperata* on bacterial isolates from blood of HIV infected patients. Methanol extract at 400 mg/ml compared favorably well with ciprofloxacin (positive control) and exhibited the highest antibacterial efficacy on *E. coli* (22.13±0.25^{h}) while chloroform extract showed the least. Table 4 shows the antibacterial activity of the purified fraction of methanol and water extract of *F. exasperata*. A 200 mg/ml of the 3rd and 4th fraction of both purified extracts recorded higher antibacterial activity against the test isolates as compared to the crude extracts. The highest activity was recorded for methanol extract against *K. pneumoniae* (32.98±0.82^{d}) while the least was aqueous extract against *E. coli* (17.96±0.33^{a}). However, purified methanol extract of *F. exasperata* showed better antibacterial activity.

Table 3. Antibacterial activity of *F. exasperata* leaf extracts on bacteria isolated from HIV infected blood

Isolates	Zones of inhibition (diameter mm)									
	Methanol (mg/ml)			Chloroform (mg/ml)			Aqueous (mg/ml)			+ve ctrl
	50	200	400	50	200	400	50	200	400	CPX
S. pyogenes	3.07±0.21^{b}	9.17±0.15^{d}	11.63±0.64^{e}	1.20±0.10^{b}	6.80±0.10^{c}	8.27±0.15^{d}	2.23±0.31^{b}	6.10±0.20^{c}	10.00±0.17^{e}	11.67±0.67^{e}
S. pneumoniae	0.00±0.00^{a}	7.90±0.10^{c}	15.77±0.25^{f}	1.10±0.17^{b}	8.13±0.15^{d}	11.67±0.21^{d}	1.90±0.17^{b}	6.93±0.12^{c}	14.40±0.27^{e}	15.00±0.58^{f}
S. aureus	2.90±0.10^{b}	15.07±0.21^{f}	19.93±0.15^{g}	1.13±0.58^{b}	9.07±0.15^{d}	10.10±0.20^{d}	3.10±0.20^{c}	10.03±0.21^{d}	18.43±0.47^{f}	10.00±0.58^{d}
P. mirabilis	3.00±0.27^{c}	10.90±0.10^{e}	18.10±0.20^{g}	1.33±0.58^{b}	7.87±0.21^{c}	12.27±0.15^{e}	2.93±0.25^{b}	9.97±0.21^{e}	16.87±0.21^{f}	14.67±0.33^{e}
S. typhi	1.77±0.15^{b}	11.13±0.21^{f}	16.07±0.25^{f}	1.23±0.58^{b}	6.10±0.10^{c}	8.90±0.17^{d}	1.60±0.10^{b}	9.00±0.10^{d}	14.07±0.15^{e}	20.00±0.00^{g}
Shigella sp	1.13±0.58^{b}	8.10±0.17^{d}	11.30±0.61^{e}	0.00±0.00^{a}	3.93±0.15^{b}	7.37±0.15^{d}	0.00±0.00^{a}	5.97±0.12^{c}	9.83±0.25^{d}	20.67±0.33^{g}
E. coli	1.10±0.17^{b}	6.23±0.35^{c}	22.13±0.25^{h}	0.00±0.00^{a}	5.70±0.25^{c}	10.73±0.15^{e}	2.07±0.15^{b}	10.80±0.20^{e}	16.13±0.21^{f}	22.33±0.33^{h}
K. pneumoniae	3.10±0.20^{c}	11.07±0.21^{e}	15.90±0.10^{f}	1.30±0.17^{b}	6.93±0.15^{d}	11.37±0.21^{e}	1.90±0.17^{b}	9.83±0.15^{e}	16.70±0.53^{f}	15.33±0.33^{e}
P. aeruginosa	1.97±0.21^{b}	10.93±0.15^{e}	21.97±0.21^{h}	1.83±0.58^{b}	6.77±0.15^{d}	10.93±0.15^{d}	1.93±0.15^{b}	10.70±0.17^{e}	16.37±0.12^{f}	19.67±0.67^{f}

Data are presented as Mean ± S.E (n=3). Values with the same superscript letter(s) along the same column are not significantly different (P<0.05)
Keys: ciprofloxacin (CPX) (10 µg), +ve ctrl = Positive control

Table 4. Antibacterial activity of purified leaf extract of F. exasperata at 200 mg/ml on bacterial isolates from blood of HIV infected patients

Isolates	Methanol extract (mg/ml)		Aqueous extract (mg/ml)	
	Fraction 3	Fraction 4	Fraction 3	Fraction 4
Shigella sp	21.23±0.33^{c}	22.12±0.19^{d}	21.67±0.87^{c}	23.07±0.69^{b}
S. pyogenes	19.96±0.33^{a}	21.07±0.66^{a}	22.92±0.66^{c}	25.02±0.69^{a}
P. aeruginosa	23.46±0.31^{c}	25.76±0.33^{c}	23.96±0.33^{a}	26.07±0.30^{a}
S. pneumoniae	20.96±0.66^{c}	21.00±0.67^{a}	21.92±0.66^{c}	22.02±0.69^{d}
P. mirabilis	24.92±0.21^{e}	25.00±0.19^{e}	26.77±0.54^{c}	27.97±0.55^{c}
S. typhi	22.96±0.10^{e}	23.22±0.87^{b}	23.92±0.66^{a}	24.02±0.69^{a}
S. aureus	26.12±0.50^{e}	26.92±0.68^{e}	28.60±0.87^{c}	29.98±0.99^{c}
K. pneumoniae	29.04±0.01^{f}	32.98±0.82^{d}	30.18±0.01^{d}	30.15±0.31^{f}
E. coli	27.92±0.66^{b}	30.02±0.69^{b}	17.96±0.33^{a}	19.00±0.67^{a}

Data are presented as Mean ±S.E (n=3). Values with the same superscript letter(s) along the same column are not significantly different (P<0.05)

3.5. Plasmid profiling of multi drug resistant bacterial isolates.

The agarose gel electrophoresis of the plasmid DNA of the multi-drug resistant bacterial isolates is shown in Figure 2. Multiple bands were observed in each bacterial lane, showing that they possessed heavy plasmids, with the highest molecular weight of 23,130 bp. However, following plasmid curing, most of the isolates were not plasmid based, as resistance was still observed in almost all the tested isolates against the antibiotics as shown in Table 5.

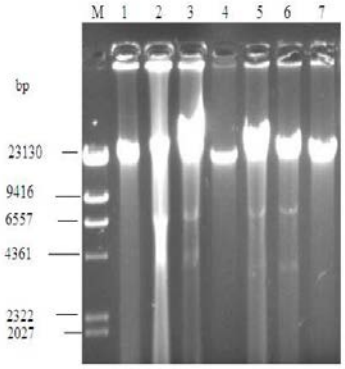

Figure 2. Plasmid profile of some selected multiple antibiotics resistant bacterial isolates from HIV infected blood samples. M: Marker, lane 1:*S. aureus*, lane 2: *P. aeruginosa*, lane 3: *K. pneumoniae*, lane 4: *E. coli*, lane 5: *P. mirabilis*, lane 6: *S. pneumoniae*, lane 7: *S. pyogene.s*

Table 5. Antibiogram profile of post cured bacteria isolates from blood of HIV infected patients

Isolates	Post cured resistant antibiotics
S. pyogenes	ERY, STR, GEN, AMX, PEF, COT
S. pneumoniae	ERY, COT, CPX
S. aureus	ERY, OFL, GEN, COT, STR
P. mirabilis	AUG, TET, CEF, NIT, PEF, COT, AMX
E. coli	AUG, TET, PEF, OFL
K. pneumoniae	AUG, TET, CEF, NIT, PEF, GEN, COT, OFL, AMX,
P. aeruginosa	AUG, TET, NIT, COT

Keys: cotrimoxazole (COT), ciprofloxacin (CPX), tetracycline (TET), amoxicillin (AMX), ofloxacin (OFL), augmentin (AUG), nitrofurantoin (NIT), gentamycin (GEN), ceftriaxone (CEF), pefloxacin (PEF), streptomycin (STR), and erythromycin (ERY)

4. Discussion

Bacteraemia is a common problem associated with HIV infected individuals, and this is usually associated with poor prognosis. Adeleye *et al*. (2008) reported that bloodstream infections constitute a significant public health problem and represent an important cause of morbidity and mortality in HIV/AIDS patients. Piroon (2007) reported that in HIV infected patients, community acquired and nosocomial bacteraemia were found in 78.5% and 25.1% respectively and are responsible for the immediate cause of death in up to 32% of HIV infected patients. Different factors may be responsible for the variation in the frequency and type of bacteria isolated from different HIV patients, such variations may be due to life styles, geographical differences, and use of intravenous drugs. Piroon (2007) also reported that bacterial infections are common in HIV infected patients because of abnormalities in humoral, cellular and mucosal immunity, and thus HIV infected patients have an increased risk of bacteraemia during bacterial infection. Bloodstream infections have been observed to appear more frequently in HIV/AIDS patients.

Gram negative bacteria were the most frequent pathogens isolated during this study. This corresponds with the findings of Piroon (2007), that bacteraemia in

HIV infected patients were usually caused by Gram negative bacteria, mostly belonging to the enterobacteriaceae family findings of Oladosu *et al*. (2016), revealed that the enterobacteriaceae family is the largest and most heterogeneous group of medically significant Gram negative bacteria and are most frequently isolated in clinical samples. They have been implicated in infections such as diarrhoea, dysentery, salmonellosis, haemolytic-uremic syndrome (HUS), necrotizing enterocolitis and various nosocomial infections (Oladosu *et al*., 2016). *Pseudomonas aeruginosa* was the highest occurring bacteria isolated in this research work (18%) and it corresponds with the findings of Ana *et al*. (2015). This bacterium has emerged as one of the most common causes of Gram negative bacteraemia and pneumonia in HIV-infected hospitalized patients, and its incidence in HIV/AIDS patients appears to be on the rise, with many studies demonstrating an annual increase in cases (Hart *et al*., 2000). Infections arising from them are hard to treat due to their natural resistance to antimicrobial agents (Obritsch *et al*., 2005). Blanc *et al*. (1998) also reported that *P. aeruginosa* is responsible for 10–15% of the nosocomial infections worldwide and has become an important cause of Gram negative infection, especially in patients with compromised immune system. *Klebsiella pneumoniae* was the most resistant isolates following post curing. This might be linked to its significant proportion in hospital-acquired urinary tract infections, pneumonia, septicemias, and other soft tissue infections. They are able to spread rapidly in the hospital environment, hence leading to nosocomial outbreaks (Podchun and Ullmann, 1998). The presence of *Shigella* spp in the blood stream of subjects analysed in this study is of great clinical concern. This high frequency of Shigella could be due to poor personal hygiene of the patients, poor food handling, preparation, bad feeding habit (malnutrition), and their choices food procurement. Kotloff *et al*. (1999) also submitted that HIV infected individuals are at high risk of recurrent, severe and fatal occurrences of bacteremia due to Shigella.

The highest occurring Gram positive bacteria from this research is *S. pneumoniae* (11%) which resides asymptomatically in healthy individuals or carriers. It has also been known to cause diseases such as community acquired pneumonia, meningitis, septicemia, bronchitis, otitis media in susceptible individuals with weaker immune system such as children, the elderly and HIV infected patients (Janoff *et al*., 1992). Other encountered Gram positive bacteria in this study, including *Streptococcus pyogenes* and *Staphylococcus aureus*, have been associated with respiratory infections from contaminated surfaces. Bianca *et al*., (2010) reported that individuals with HIV/AIDS infection are also at an increased risk of *S. aureus* bacteremia whose sources might be hospital acquired, community and health care.

The multi-drug resistance displayed by most of the organisms to the various test antibiotics is a major cause for concern because many clinicians fall back on the use of quinolones for the treatment of Gram-negative pathogens in the face of multi-drug resistance (Khaneja *et al.*, 1999). The high rate of resistance towards these antibiotics may be due to drug abuse because they are readily available over the counter, not completing a dose before beginning a new one. Genetic and non-genetic acquired resistance can lead to increased bacterial antibiotics resistance (Mutation or Plasmid acquired resistance). Ciprofloxacin, which belongs to the family of quinolone antibiotics, displayed the highest antimicrobial effect of all the antibiotics used in the antimicrobial susceptibility testing; this outcome agrees with a report from Oladosu *et al*. (2016). Prajna *et al*. (2001) reported that the ophthalmic solutions of ciprofloxacin antibiotics are effective and safe in the treatment of patients with culture positive bacterial keratitis. According to studies, the use of antibiotics, especially irrationally, is the reason for the emergence and spread of resistant strains of pathogens that are of clinical importance in both the community and hospital environments (Bradford, 2001; Schmitt *et al*., 2007).

Plants have a diverse range of bioactive molecules, making them rich sources of different types of medicines. These bioactive compounds, according to Aboaba *et al.* (2006) usually interfere with the growth and metabolism of microorganisms in a negative manner.The phytochemical test carried out on the powdered leaf extract revealed the presence of phytochemicals of pharmacological importance (Adebayo *et al*., 2009). The presence of flavonoid is an indication of natural occurring phenolic compound with beneficial effects in the human diet as anti-oxidant and free radicals (Adebayo and Ishola, 2009). The presence of flavonoid, tannins, saponins, phenolic constituents could provide synergistic effect which improves the efficacy of the active ingredients of the leaf (Takon *et al.*, 2013). The leaf extract of *F. exasperata* at 200mg/ml compared favorably with the commercial antibiotics. Methanolic extracts of *F. exasperata* leaves had greater antibacterial potency against the tested isolates compared to the aqueous and chloroform extracts. The presence of high amount of flavonoid which is a common secondary metabolite in medicinal plant may have accounted for this high antibacterial efficacy. The results of these inhibition growth zones of these is a confirmation of the ethno-medicinal significance of the plant, especially against secondary bacterial infections associated with HIV infection.

All the selected multi-drug resistant isolates harbor plasmids. Plasmids are extra-chromosomal pieces of DNA which are capable of replicating independently of the genome and have been directly implicated in the acquisition of resistance to many antibiotics (Weigel *et al.*, 2003).They have been documented to have encoded gene that provides resistance to occurring antibiotics in competitive environmental niche (Kroll *et al*., 2010). The post curing sensitivity test revealed that the resistance was not plasmid mediated, indicating chromosomal-borne resistant genes. Moreover, resistance to antibiotics by bacteria that is not due to plasmid or chromosome might be due to efflux pump mechanism (Poole, 2004) or other factors like mutation of gene encoding ribosomal protein which decrease permeability of the cell envelope in enteric bacteria (Isenberger *et al*., 2002).

5. Conclusion

It is important to monitor the susceptibility patterns of microorganisms as it contributes significantly to the burden of secondary bacterial infection in HIV infected patients.The antibacterial potential of the leaf extract of *F. exasperata* may be a source of new bioactive compounds

for drug development and also suggests it as a cost effective alternative therapy against opportunistic bacteria associated with HIV. Further purification of the extract and identification of the bioactive component is necessary to enhance greater antimicrobial potency.

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The Variations in Saliva and Serum Total Peroxidases System's Activity in Patients with Different Oral Tumors

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Abstract

Salivary peroxidases have biological functions of particular importance to oral health. The aim of this paper is to shed the light on saliva and serum total peroxidases activity as well as the activity of each of salivary peroxidase (SPO) and myeloperoxidase (MPO) in patients with oral tumors. The studied participants were divided into two groups: the first group included 18 oral squamous cell carcinoma patients and 20 age and gender-matched healthy controls while the second group consisted of 20 oral ossifying fibroma patients and 23 age and gender-matched healthy controls. Total peroxidases activity was determined, and its specific activity was calculated in serum and whole mixed saliva as well as in the supernatant and pellet fractions of saliva. Furthermore, the activities of SPO and MPO were determined in each of saliva's supernatants and saliva's pellet fractions, and the thiocyanate (SCN^-) concentration was measured in the supernatants fraction only. The results indicated the presence of a significant increase in the activity of both total peroxidase and MPO ($p = 0.0001$) in the salivary supernatants of oral squamous cell carcinoma patients relative to the control group. A significant increase ($p = 0.0001$) in total peroxidase activity in patients with oral ossifying fibroma was also found in serum compared with healthy individuals. In this study, we have shown that the measurement of total peroxidase and MPO activities in saliva may be used as an adjuvant tool for monitoring patients with oral malignancies.

Keywords: Oral squamous cell carcinoma, Ossifying Fibroma, Salivary peroxidase, Myeloperoxidase, Thiocyanate.

1. Introduction

Oral cancer is one of the most common cancers in the world, with approximately 274300 new cases and 127500 deaths occurring each year. Over 90% of total oral cancers reported being squamous cell carcinomas (OSCC) (Scully, 2013). OSCC occurs in all sites of the oral cavity, but the majority of cases involve the tongue, oropharynx, and floor of mouth (Chi *et al.,* 2015). Ossifying fibroma (OF) is a true neoplasm that is composed of fibrous tissue containing a variable mixture of bony trabeculae, cementum-like spherules, or both) with significant growth potential. It has been suggested that the origin of these tumors is either odontogenic or from periodontal ligament**.** Ossifying fibroma occurs over a wide age range with the greatest number of cases encountered during the third and fourth decades of life (Neville *et al.,* 2009.).

Peroxidase enzymes have been reported to play a key role in many human diseases where the activity of these species can be both beneficial and detrimental. Generally, SPO is known to be the major peroxidase present in human saliva, while MPO is released from oral leukocytes in amounts proportional to the degree of gingival inflammation (Koss *et al.,* 2016; Moriguchi *et al.,* 2017). The two types of peroxidases enzymes are distributed unevenly between the pellet and the supernatant fraction of saliva (Thomas *et al.,* 1994).

Due to the very limited studies on SPO and MPO activities in serum and saliva of patients with different oral tumors, we previously studied this enzymatic system using electrophoresis as a separating tool (Hasan and Aburahma, 2014.). The electrophoretic profile of the total peroxidase system showed the appearance of a new band that was stained for peroxidase activity in the saliva of Iraqi patients with OSCC. Therefore, the present study aims at highlighting the variations in the activity of this system in saliva and serum of the two most common types of oral tumors in Iraq, OSCC and OF.

2. Materials and Methods

2.1. Studied groups

A total of 38 non-alcoholic and non-smoking patients with primary oral tumors, who were attending the Department of Oral and Maxillofacial Surgery (Al- Wasity Hospital and Hospital of Specialized Surgeries, Medical city**,** Baghdad, Iraq) were included in the current study. The samples were collected from these patients during the period from May to November of 2016. Patients who were presented with distant metastases, patients who underwent radiotherapy and those whose saliva samples were mixed with blood were excluded from the study. Moreover, according to the type of tumor, the patient's groups were divided into two groups: The first group included 18 patients with OSCC while the second group included 20 patients with oral OF.

For comparison purpose, 43 non-alcoholic and non-smoking healthy, age and gender matched, volunteers were

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used as a control, where 20 of them were used as a control for the first group of the patients and 23 individuals for the second group. The protocol of the present study was approved by the Ethics Committee of the College of Science, University of Baghdad, Baghdad, Iraq.

2.2. Saliva samples

Unstimulated 5 to 10 mL whole, mixed-saliva samples were collected on ice, under resting conditions in a quiet room between 8.0-9.0 A.M. The Patients and healthy individuals were asked to rinse their mouth with normal saline, then to generate saliva in their mouth and to spit into a plastic container for 10 minutes. After collection, the saliva was immediately divided into two test tubes, the first one was labeled as "total saliva" and stored frozen, while the remainder saliva sample (3 mL) was centrifuged at (2000 ×g) for 10 minutes at 4℃ to obtain the supernatant and the pellet fractions. The obtained fractions were stored frozen at –20℃ in polyethylene tubes until used for the different assays. Just before analysis, the peroxidases were extracted from the pellet fractions using phosphate-buffered saline 0.2M; pH 7.0 containing 0.1% cetyl-tri-methyl ammonium bromide (CTAB) as reported by Thomas *et al.* (Thomas *et al.,* 1994). The pellet was washed with cold phosphate-buffered saline and centrifuged at (2000 ×g) for 5 minutes at 4℃. Then the supernatant fraction was discarded and the pellet was suspended to the original volume of cold phosphate-buffered saline 0.2M; pH 7 containing 0.1% CTAB in an ice bath and sonicated at 10 µm Amplitude for 1 minute at intervals of 15 second using MSE SONIPREP 150 and incubated for one hour at 4℃ to complete the extraction. Finally, the sample was centrifuged for 10 minutes at (3000 ×g) and the supernatant was used for protein and enzyme activity measurements on the same day.

2.3. Serum Samples

Ten milliliters of venous blood samples were collected using plastic disposable syringes from overnight fasting patients (before surgery) and the control groups, in plain polyethylene tube. The blood samples were allowed to clot for ten minutes at 37℃ in a water bath, then they were centrifuged at (3000 ×g) for 10 minutes. The obtained clear serum supernatant was stored frozen at -20℃ until being assayed for the different parameters.

2.4. Determination of total peroxidases

Total peroxidases activity was measured in saliva and serum, as well as its distribution between the saliva's supernatant and pellet fractions by colorimetric method using phenol, 4- aminoantipyrine and H_2O_2 as the dye-generating compounds (Song *et al.,* 2005). The activity was expressed as the increase in absorbance at $\lambda = 510$ nm resulting from the decomposition of hydrogen peroxide per time of incubation (ΔA/min) using a molar extinction coefficient of 7100 $M^{-1}cm^{-1}$. The specific activity is expressed for all studied enzymes as a unit of enzymatic activity/mg of protein concentration, where the protein concentration was estimated as previously described using a modified Lowry method (Hasan and Abdelwahab, 2014).

2.5. Determination of salivary peroxidase and myeloperoxidase

In order to be able to determine each of SPO and MPO separately, the saliva samples were desalted using PD10 column to remove the SCN^- and chloride ions (Cl^-) from the saliva samples (Helmerhorst and Stokes, 1980). Then, SPO and MPO activities were determined using the method described in Mansson-Rahemtulla *et al.,*1986. Briefly, this method was based on the oxidation of the intensely yellow 5-thio-2-nitrobenzoic acid (TNB) to the colorless 5, 5'-dithiobis (2-nitrobenzoic acid) (DTNB) by $OSCN^-$ ions generated during the oxidation of SCN^- . The presence of Cl^- only in the assay mixture makes the method suitable for the determination of MPO activity, since Cl^- is oxidized to OCL^- by this type of peroxidase, but not by the SPO type (Kaczmarek, 2005). One unit of enzyme activity was expressed as the level of enzyme activity needed to cleave 1 µmol of TNB/min at 22ºC, using a molar extinction coefficient of 12.800 $M^{-1}cm^{-1}$ (Goi *et al.,* 2007).

The activity of SPO = Total peroxidases activity – MPO activity

2.6. Determination of salivary thiocyanate (SCN-) concentration

Salivary thiocyanate concentration was measured spectrophotometrically as described by Aune and Thomas (Aune and Thomas, 1977). This concentration (expressed in mM) in saliva's supernatant fractions was calculated using the linear equation derived from the standard curve which was constructed by plotting the absorbance at 450 nm of the produced $FeSCN^{2+}$ in presence of different concentrations of KSCN (ranging between 0.2-2.4 mM) treated as above.

2.7. Statistical Analysis

Statistical analysis was carried out using the program Statistical Package for the Social Science (SPSS for Windows, version 21 software packages). The differences between groups were tested by the Student t-test, and the *p*-value was considered significant if it was < 0.05.

3. Results

The baseline characteristic of the studied groups is illustrated in Table 1. The study population consisted of a total of 38 patients including 18 with OSCC and 20 with OF: the age ranges were (59.2±11.6 and 23.4±16.0) respectively, with two groups of healthy individuals serving as control corresponding to each patients group who were within about the same age range.

Table 1. Characteristics of the studied population.

Group	Sub-Group	Age (year) (Mean±SD)	Gender	Site of tumors	Histopathological Type	Clinical staging of patients	Histopathological grading
First group	Control (n=20)	56.5±10.2	10 males 10 females	-	-	-	-
	Malignant (OSCC) (n=18)	59.2±11.6	10 males 8 females	Tongue	Squamous cell carcinoma	Stage II	Grade II - Moderately –well differentiated
Second Group	Control (n=23)	26.0±12.2	10 males 13 females	-	-	-	-
	Benign (OF) (n=20)	23.4±16.0	6 males 14 females	jaw	Ossifying fibroma	-	-

3.1. Total salivary peroxidase activity and specific activity

The results of total peroxidase activity's measurement in the saliva samples of all studied groups and the calculation of the specific activity are presented in Table 2.

Table 2. Total peroxidase activity and specific activity in the salivary samples of patients with different oral tumors OSCC and OF: first and second groups, respectively.

Group	Sub-Group	Activity (U/L) *P*-value			Specific Activity (U/g) *P*-value		
		Total saliva	Supernatant of Saliva	Pellet of saliva	Total saliva	Supernatant of saliva	Pellet of saliva
First group	Control (n=20)	365.8±118.5	125.5±53.3	147.6±59.5	142.2±31.4	59.6±20.0	599.7±107.4
	Malignant (OSCC) (n=18)	485.7±186.1*	240.7±99.7*	141.3±59.4	110.8±26.0*	101.2±37.0*	329.9±183.4*
		0.023	0.0001	0.750	0.002	0.0001	0.0001
Second group	Control (n=23)	347.4±97.6	112.3±40.7	148.2±59.9	116.1±43.1	55.4±17.3	417.7±131.6
	Benign (OF) (n=20)	411.7±199.3	199.9±87.6*	107.9±48.3*	104.5±42.7	88.4±30.9*	288.0±71.5*
		0.179	0.0001	0.021	0.380	0.0001	0.001

* Significant difference in comparison to control at ($p < 0.05$).

From the results presented in the table 2 above, a significant increase ($p < 0.05$) in salivary total peroxidase activity and specific activity of malignant tumors (OSCC**)** patients in comparison to that of healthy control was observed. In the benign tumors (OF) patients' group, however, the increase in total saliva peroxidase activity in comparison to that of healthy controls was non-significant ($p > 0.05$).

Upon calculating the percentage of peroxidase activity present in the salivary supernatant and pellets in relation to the activity of peroxidase in total saliva, the percentage of distribution of this activity appeared to be as follows: 49.8% and 30.5% in the supernatant and pellet fractions respectively in malignant tumors patients' group (OSCC**)** and 50.5% and 27.7% respectively in benign tumors patients' group (OF).

3.2. Serum peroxidase activity and specific activity.

The activity of total peroxidase was measured in the serum samples as described in the methods section and its specific activity was calculated. The results in Table 3 reveal the presence of a significant increase ($p < 0.05$) in serum peroxidase activity of the (OF) group in comparison with that of their corresponding control. On other hand, the increase in this activity was observed to be non-significant ($p > 0.05$) in sera samples of (OSCC**)** patients in comparison with that of the corresponding control group.

Table 3. Total peroxidase activity and specific activity in the serum samples of patients with different types of oral tumors; OSCC and OF.

Group	**Sub-Group**	**Total serum peroxidase**	**Activity (U/L)**	**Specific Activity (U/g)**
	Control (n=20)	Mean±SD	49.8±20.5	0.7±0.3
	Malignant (OSCC) (n=18)	Mean±SD	64.0±36.6	0.9±0.6
		P-value	0.135	0.107
	Control (n=23)	Mean±SD	30.4±9.5	0.4±0.1
	Benign (OF) (n=20)	Mean±SD	39.0±11.9*	0.5±0.1*
		P-value	0.012	0.0001

* Significant difference in comparison to control at ($p < 0.05$).

3.3. Evaluation of salivary peroxidase and myeloperoxidase activities and specific activities

The results in Tables 4A indicate the presence of a significant increase ($p < 0.05$) in MPO activity and specific activity in the salivary supernatant fractions of both studied groups in comparison to that of their corresponding healthy controls. In contrast, there is a non-significant ($p > 0.05$) slight decrease in the activity of SPO in the saliva samples of all patient's groups compared with their corresponding control groups. On the other hand, there is a significant decrease in SPO specific activity ($p < 0.05$) in patients with malignant tumors (OSCC) in comparison to that of healthy controls.

Table 4. SPO and MPO activities and specific activities in the salivary supernatant and pellet of patients with different oral tumors OSCC and OF: A: in the supernatant fraction of saliva and B: in the pellet fraction of the saliva.

A: Supernatant fraction.

Group	Sub-Group	Activity (U/L) *P*-value			Specific Activity (U/g)*P*-value		
		Total peroxidases (SPO+MPO) (U/L)	MPO (U/L)	SPO (U/L)	Total peroxidases (SPO+MPO) (U/L)	MPO (U/L)	SPO (U/L)
First group	Control (n=20)	212.8±42.7	23.5±8.9	189.3±37.3	106.4±42.5	11.9±3.9	95.2±26.2
	Malignant	218.9±70.3	58.9-21.5*	161.5±56.4	85.8±31.4	23.7±11.9*	62.8±24.9*
	(OSCC) (n=18)	0.748	0.0001	0.082	0.1	0.0001	0.0001
Second group	Control (n=23)	185.8±78.8	17.1±7.5	168.7±72.7	95.1±31.5	9.0±3.7	84.2±29.4
	Benign (OF) (n=20)	193.5±19.0	29.8±11.9*	163.7±22.3	93.9±34.3	13.5±3.9*	80.6±32.5
		0.672	0.0001	0.772	0.904	0.0001	0.705

B: Pellet fraction.

Group	Sub-Group	Activity (U/L) *P*-value			Specific Activity (U/g) *P*-value		
		Total peroxidases (SPO+MPO) (U/L)	MPO (U/L)	SPO (U/L)	Total peroxidases (SPO+MPO) (U/L)	MPO (U/L)	SPO (U/L)
First group	Control (n=20)	145.9±60.9	30.8±11.1	118.3±56.2	627.4±154.9	118.8±65.9	527.4 ±141.1
	Malignant	117.6±71.1	31.2±12.8	86.4±60.0	253.6±65.6*	79.7±36.8	188.6±67.7*
	(OSCC) (n=18)	0.202	0.915	0.104	0.0001	0.095	0.0001
Second group	Control (n=23)	190.6±84.7	48.4±23.9	142.1 ±66.3	572.9±220.9	143.5±69.4	431.6±185.3
	Benign (OF) (n=20)	224.6±98.4	96.9±48.8*	127.7±66.3	621.1±202.2	307.3±132.3*	338.1±105.2
		0.231	0.0001	0.482	0.463	0.0001	0.053

* Significant difference in comparison to control at ($p < 0.05$).

Regarding SPO in the pellet fraction of the saliva, the results from Tables 4B indicate the presence of a significant decrease in this enzyme specific activity ($p < 0.05$) in the (OSCC**)** patients upon comparison with that of its control group. Meanwhile, the activity and specific activity of (MPO) in this type of disease show a non-significant difference ($p > 0.05$) in comparison with that of its healthy controls.

In the patients with benign bone tumors group (OF), a significant increase ($p < 0.05$) in MPO activity and specific activity compared with healthy controls was recorded while for SPO activity and specific activity, a non-significant decrease ($p > 0.05$) in patients group compared with control is observed.

3.4. Determination of thiocyanate (SCN-) Concentration

When the SCN^- concentration in the saliva supernatant was estimated as described in the method and material section, the results reveal that the SCN^- concentration is significantly lower ($p = 0.003$) only in (OF) patients in comparison with that of the control Table 5.

Table 5. SCN^- concentration in salivary supernatant of patients with different oral tumors OSCC and OF: first and second groups, respectively.

SCN conc. (mM)	First group		Second group	
	Control (n=20)	Malignant (OSCC) (n=18)	Control (n=23)	Benign (OF) (n=20)
Mean±SD	0.6±0.2	0.4±0.2	0.6±0.3	0.3±0.3*
P-value	-	0.108	-	0.003

* Significant difference in comparison to control at ($p < 0.05$).

4. Discussion

The reported relative contributions of SPO and MPO activities in salivary total peroxidase activity were variable and sometimes contradictory, Klein *et al* and Nagler and Reznick using 2-nitrobenzoic acid-thiocyanate assay for measurement of total peroxidase and relative contribution of SPO and MPO in this total activity have concluded that the SPO is secreted from the major salivary glands, mainly from the parotid gland, and contributes approximately 80% to total peroxidase activity, whereas MPO, produced by leukocytes in inflammatory regions of the oral cavity, contributes the remaining 20% of total peroxidase activity (Klein *et al.*,2003; Nagler and Reznick, 2004). On the other hand, Thomas *et al.*, using 4-aminoantipyrine as a substrate mentioned that MPO is responsible for an average of 75% of the total peroxidase activity in the mixed saliva. The two types of peroxidases enzymes are distributed unevenly between the supernatant and the pellet fraction of saliva. SPO is found in the soluble portion and almost 80% of MPO is found in the salivary pellet (Thomas *et al.,* 1994). Unraveling this contradiction has been tested throughout the present study by measuring the relative contribution of SPO and MPO to total peroxidase activity in different salivary components, and the following results were found: in the control of the first group (Table 4) the percentage of the activities of SPO and MPO activities were 88.93% and 11% respectively in the salivary supernatant fractions. Meanwhile, approximately the same percentage is calculated in the control of the second group (90.7% and 9.2% for SPO and MPO, respectively) (Table 4 A), whereas in the salivary pellet fractions the percentages of SPO and MPO are found to be 81% and 21%, respectively, in control of the first group while they were found to be 74.5% and 25.4%, respectively in control of the second group (Table 4B). Based on these results, it can be concluded that SPO activity is the major component of total salivary peroxidase in both the supernatant and pellet of saliva. This agrees with other studies on saliva peroxidase activity of normal healthy individuals (Nickerson *et al.*, 1957; Iwamoto *et al.*, 1968; Thomas *et al.,* 1994).

The relationship between MPO and different types of tumors has not been clarified and thus needs intense research (Al-Salihi *et al.*, 2015). Furthermore, MPO has been reported to produce biochemical alterations in different antioxidative species that lead to cancer progression (Khan *et al*., 2018), and since the studies on salivary MPO in oral tumors is very limited, the aim of this study, therefore, was to investigate the alteration of MPO in serum and saliva of OSCC and OF patients.

The observed increase in MPO (Table 4) disagrees with the only study found in the literature by Ajila *et al.*, 2015 which refers to non-significant decreased MPO activity in saliva of OSCC Indian patients. On the other hand, the observed elevation of the MPO activity in saliva of present studied patients' groups agrees with some studies in different oral diseases other than tumors that have demonstrated an increase in MPO levels and/or activity in the gingival crevicular fluid (GCF) of patients with periodontitis (Karhuvaara *et al.*, 1990; Yamalik *et al*.,2000; Wei *et al.*, 2004; Borges *et al.*, 2007).

The obvious elevation in this study of MPO activity in saliva of patients with oral tumors may be related to the elevation in serum peroxidase and may give a clue that the measured serum peroxidase in our study is more or less contributing to salivary MPO activity. This type of peroxidase in the oral cavity mostly comes from a gingival crevicular fluid (GCF) (Bafort *et al.*, 2014), and gingival crevicular fluid is a serum transudate: the fluid passes from the systemic circulation through the junctional epithelium of the gingiva and into the gingival crevice/oral cavity (Davies and Finlay, 2005).

On the other hand, the observed decreased SPO activity in the saliva samples seems to agree with the findings of Bahar *et al*., (2007) who had found that all salivary antioxidants, including salivary peroxidase, were substantially reduced in patients with oral squamous cell carcinoma. They reported that this decrease was due to the depletion of salivary antioxidants systems as a result of the increase in reactive oxygen species (ROS) and reactive nitrogen species (RNS). Such a state explains the oxidation of DNA and proteins and the promotion of OSCC. Cancer has been recognized as a form of chronic inflammation, involving the participation of large pool cell types (Wang *et al.*, 2020). The increased free radicals (which are highly reactive chemical compounds that has one, or more unpaired valence electrons) (Sharma, 2014) are well-known inducers of cellular and tissue pathogenesis leading to numerous disorders including tumors; such free radicals are normally scavenged by antioxidant enzymes among which salivary peroxidase is one of the most important ones present in submandibular glands. This antioxidant system acts to prevent the negative effect of these free radicals in causing the appearance of cancer (Filip *et al*., 2007). It was reported that at the time of malignant tumor setting, the reduction in SPO activity was highest and such a reduction may result from an increase in the level of oxidants (Bentur *et al*., 2006) and may be of paramount importance since SPO has a dual role. First, it controls the level of H_2O_2 excreted by bacteria and leukocytes from the salivary glands into the oral cavity (Nagler *et al*., 2002) by a mechanism that protects oral mucosa from cellular lysis induced by H_2O_2 (Filip *et al*., 2007). Meantime, H_2O_2 is considered as ROS which plays a key role in human cancer development since this compound with other free radicals can cause DNA base alterations, strand breaks, damaged tumor suppressor genes, and an enhanced expression of protooncogenes. ROS induced mutation could also result in protein damage (Bahar *et al.*, 2007). The second role of SPO is its specific antibacterial activity that inhibits the metabolism and proliferation of various bacteria in the oral cavity (Nagler *et al.*, 2002). It is known that peroxidase activity is a marker of salivary glands functionality and that ''*in vivo*'' salivary peroxidase catalyzes the oxidation of the SCN^- ion to $OSCN^-$ ion and hypothiocyanous acid (HOSCN), The latter compound inhibits the growth and metabolism of many species of pathogens. Moreover, the salivary peroxidase system maintains the thiocyanate peroxidation reactions in a state of ''*in vivo*'' dynamic equilibrium thereby minimizing the concentration of toxic H_2O_2 and maximizing the concentration of the antibacterial agent hypothiocyanite (Filip *et al.*, 2007).

The complete antimicrobial peroxidase system consists of peroxidases together with the naturally occurring SCN^- and H_2O_2 (Tonoyan *et al.*, 2017). SCN^- reduces certain tissue-damaging species [e.g., H_2O_2 and hypochlorite (OCl^-)] by subjecting itself to oxidation. In the absence of adequate SCN^-, overproduction of OCl^- by MPO during inflammation might result in severe injuries and lead to the self-destruction of white blood cells. The death of these cells would in turn cause additional destructive agents to be dumped, escalating injuries to the host. The measured decrease in the concentration of SCN^- in the current study provides inadequate protection against the overproduction of OCl^-, thus worsening inflammatory diseases and predisposing to diseases linked to MPO activity including cancer (Xu *et al.*, 2009). The development of cancer depends on the extent of DNA damage; this damage is proportional to the magnitude of oxidative and nitrative stress that reflect the net effect of both ROS and RNS, as well as the effectiveness of the antioxidant's defense system and the DNA repair system. ROS and RNS are involved in the initiation and promotion of cancer, and they are inhibited by antioxidants, however, when the equilibrium is broken DNA is oxidized and cancer evolves (Bahar *et al*., 2007).

In conclusion, although the size of the samples is limited due to the exclusion of many saliva samples of patients with OSCC because of the presence of blood mixed with the saliva, the main interesting results of the current study were the finding out of an elevation in salivary total peroxidases and myeloperoxidase activities in the patients with oral OSCC. The measurement of these activities in saliva may be proposed as an adjuvant tool for monitoring the patients for the presence of OSCC, a role which needed further study with a larger number of samples in order to be confirmed.

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Effects of Inoculation with Arbuscular Mycorrhizae and Ectomycorrhizae on Growth and Mycorrhizal Colonization of Cork Oak (*Quercus suber* L.)

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Abstract

The cork oak (*Quercus suber* L.) is a tree which forms important forests that play a vital socioeconomic and environmental value in the Mediterranean basin. The multiple factors of overgrazing, biological invasion by introducing acacia trees, and repeated fires weaken the cork oak ecosystem and affect its natural regeneration. The purpose of this study is to increase the production of cork oak. With this aim, a test of controlled mycorrhization was carried out on the nursery where two commercial inocula—the arbuscular mycorrhizal (Symbivit) (S) and ectomycorrhizal (Ectovit) (E)—were brought separately and combined on cork oak seedlings cultivated on sterilized or non-sterilized soil. Statistical models revealed that the controlled inoculation improved the growth of the plants inoculated by IE, which had significantly increased mycorrhizal root colonization levels and cork oak growth compared to the treatments of the Symbivit (IS) and the non-inoculated plants (NI); these rates were lower in both substrates: sterile and non-sterile. It is known that the best mycorrhizal partners of cork oak are ectomycorrhizae, however, in the presence of arbuscular mycorrhizae, the mycorrhizal root colonization levels and the growth parameters were considerably enhanced compared to previous treatments IE, IS and NI. The dual colonization had shown positive effects on the improvement of the mycorrhizal potential of the soil. Indeed, EM % colonization was the most strongly correlated with growth parameters compared to other mycorrhizal parameters. This research underlines that the use of controlled inoculation based on commercial inoculum can be an effective alternative in the case where the local inoculum is not available, and thus time saving.

Keywords: *Quercus suber*, Arbuscular mycorrhizae, Ectomycorrhizae, Dual colonization, Growth, Mycorrhizogenic potential

1. Introduction

Cork oak (*Q. suber*) woodlands are ecosystems of high environmental and socioeconomic values, characterized by a vegetation cover that supports high levels of biodiversity (Bugalho *et al*., 2011). Native cork oak forests occupy 1.3 million ha in southern Europe (Portugal, Spain, France and Italy) and 0.9 million ha in North Africa (Morocco, Algeria and Tunisia) (Lancellotti and Franceschini, 2013). The cork oak is threatened due to the combined effects of overexploitation of wood, overgrazing, deforestation, and the repeated fires (Lancellotti and Franceschini, 2013). However, the cork oak decline has multiple impacts at above ground and below ground levels, strongly affecting resilience and productivity of cork oak forests (Maghnia *et al*., 2017).

Natural regeneration from seed is not always successful, and the survival rate of transplanted seedlings is often low (Sebastiana *et al*., 2013). For this purpose, an exploration of the potential of the association between *Q. suber* and beneficial microbial symbionts can be the crucial solution to produce high-quality seedlings of cork oak in the nursery stage (Araújo *et al*., 2018).

Mycorrhizal fungi are ubiquitous components of most ecosystems throughout the world and are considered key ecological factors in biological processes (Schreiner *et al*., 1997), increasing plant tolerance to environmental stresses (Meddad-Hamza *et al*., 2010), promoting plant growth in soils with low water and mineral availability (Bingham and Simard, 2012), allowing seedlings survival (Wezowicz *et al*., 2017), reducing soil fertilization and irrigation requirements (Rillig *et al*., 2015), and contributing to the restoration of degraded soil (Asmelash *et al*., 2016).

Cork oak species form a dual symbiotic association with arbuscular (AMF) and ectomycorrhizal fungi (EMF) (Hamidi *et al*., 2017). Thus, in the objective to improve the quality and ecosystem resilience of nursery-produced *Q. suber* seedlings, the association with ectomycorrhizal and arbuscular fungi should be a forefront strategy (Araújo *et al*., 2018).

Thus, the main objective of this ecophysiological study is to highlight the beneficial effects that represent different AMF and EMF inoculations for the cork oak. For that, an arbuscular mycorrhizal and an ectomycorrhizal commercial inoculum (Symbivit and Ectovit respectively), were used separately or in combination. Concurrently, the objective of these root inoculations with different AMF and EMF strains is to determine the efficacy and infectivity of these both strains for cork oak.

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2. Materiels and Methods

2.1. Inoculation of Q. suber seedlings

Two inoculants were tested, a commercial arbuscular mycorrhizal inoculum Symbivit® and ectomycorrhizal fungi (EMF) Ectovit® **(**INOCULUM plus, France**)**.

Symbivit® granulated and full of 6 AMF species which are composed of natural clay and propagules (*Claroideoglomus etunicatum*, *C. claroideum*, *Glomus microaggregatum*, *Rhizophagus intraradices*, *Funneliformis mosseae*, *F. geosporum*) where we have used a number of 200 propagules per plantlets While Ectovit® contained propagules of 6 different species of EMF; 4 strains of mycorrhizal fungi on a liquid medium agar (*Amanita rubescens*, *Hebeloma velutipes*, *Laccaria proxima*, *Paxillus involutus*), and 2 strains of mycorrhizal fungi (*Pisolithus arrhizus*, *Scleroderma citrinum*) on a peat-based carrier with a minimum number of spores of 300 million per gram of dry matter.

The arbuscular mycorrhizal inoculum (Symbivit) is mixed with the substrate at the rate of 10 g of inoculum, while the ectomycorrhizal inoculum (Ectovit) was brought from the second month of growth as a paste prepared by mixing fungal mycelium with dry inoculum components (including fungal spores) and sufficient water. The mixture was subsequently distributed in six 10 cm deep holes, dug using a glass bar closest to the roots of each cork oak plant.

2.2. Experimental design

The experiment was conducted in station of the National Institute of Forest Research (NIFR) at the nursery of Guerbes (36°56'6.79'' N; 7°11'18.36" E), Skikda region, from 14 April 2014 to 14 January 2015, under natural daylight. The daily average temperature was 10.8-28.8 °C and relative humidity of 60-70%. This nursery is specialized in the production of forest plants, especially cork oak, intended for the reforestation of the Northeastern region of Algeria.

The culture substrate used in this experiment represents a mixture of 60% olive pomace and 40% forest humus. Olive pomace is the waste recovered from oil-mills that has undergone composting for three years in order to reduce the levels of acids and toxic compounds that may be present. The forest humus originates from the decomposition of the accumulated matter under a vegetation of cork oak forests of Guerbes. A portion of the substrate sample was sterilized (SS) twice in an autoclave, 20 min at 120°C with 24 h between each autoclave, and the rest was not sterilized (NS). *Q. suber* acorns were collected directly from one tree in November 2014 in the Brabtia nature reservation (36°52'09.27''N; 8°20'16.58''E), located in the commune of El Kala, in the Northeast of Algeria. The acorns were washed with tap water and a few drops of detergent disinfected by immersion (15 min) in a solution of 30% hydrogen peroxide. They were then rinsed several times with sterile water, later placed to germinate during ten days under aseptic conditions in wet soil previously autoclaved twice for 20 min at 120 °C and then stored in a culture chamber under strict conditions: temperature 20 °C, controlled humidity, and darkness.

The sterile (SS) and non-sterile (NS) substrates are dumped into WM containers at 900 g per container with a pregerminated acorn. The experimental setup was simple randomization with 8 treatments repeated 7 times for a total of 56 plants: cork oak seedlings inoculated (I) by Ectovit (E), Symbivit (S), the combination of both E and S that became (M) grown on sterile (SS) and non-sterile (NS) substrate. Two control non-inoculated (NI) grown on SS and NS were made. The plants were irrigated with an automatic system three times a week. This frequency starts daily in the summer and then decreases during the wet months.

2.3. Plant sampling and analyses

After 9 months of growth**,** plant height, shoot and root fresh weight and the mycorrhizal root colonization levels of the cork oak plants were estimated. Moreover, an evaluation of the content of the leaf chlorophyll was assessed with Chlorophyll Meter Konica Minolta SPAD-502 Plus. The SPAD values were taken at the top, the middle and the base of the leaf. The chlorophyll value obtained in the SPAD unit is the average of the three values read on the screen.

Growth percentage of all parameters was calculated by the use of the following formula: Growth increase (%) = [(growth of inoculated plants - growth of non-inoculated plants)/growth of non-inoculated plants)] × 100 (Plenchette *et al.*, 1983).

2.4. Assessment of EM root colonization

Ectomycorrhizal (EM) colonization assessment was determined by counting the presence or the absence of colonized root tips under a stereomicroscope according to the method of Brundrett *et al*., 1996. The percentage of root colonization was determined for each sample by examining 300 1 cm-long pieces of root, expressed with the following formula:

EM colonization rate (%) = [Number of mycorrhizal root pieces /Total number of observed root pieces] x100.

2.5. Determination of arbuscular mycorrhizal colonization

Arbuscular mycorrhizal (AM) root colonization was estimated on the basis of the Phillips and Hayman (1970) method. Samples were processed, undergoing the following stages: soil washing, cutting into segments of 1.5 cm, hot cleaning with KOH 10% (15 min), immersion into a solution of HCl 20% (10 min) and staining with 0.03% Trypan blue solution at 90 °C.

2.5.1. Estimation of root mycorrhization

Annotation was made according to the method described by Trouvelot and Kough (1986), which is a fast-technique reflecting as much as possible the potential and the state of activity of mycorrhizal symbiosis. Root observations were done for 5 repetitions of 30 root fragments of 1 cm, placed between slides and lamellae and observing them under a light microscope. The operation was repeated twice to calculate five mycorrhization parameters using the MycoCalc computer program (http://www2.dijon.inra.fr/mychintec/).

2.6. Mycorrhizogenic potential of soil

AMF propagules were assessed after inoculation using the most probable number (MPN) method (Porter, 1979), which is based on the use of a series of successive soil dilutions at the rate of 10 (1/10, 1/100, 1/ 1000, 1/10,000

and 1/100,000) for determining the limiting dilution at which no AMF propagules can be detected. The dilutions were prepared by mixing the original soil with the same soil autoclaved twice at 120 °C (Gianinazzi-Pearson *et al*., 1985). The soil mixture is divided into five replicates of 50 g per pot. Pregerminated clover (*Trifolium repens* L.) seeds were planted with one seedling per pot. The seedlings were transferred under controlled conditions in a greenhouse (average daily temperature 18–22 °C, with 60–70% relative humidity) and watered daily with distilled water. After six weeks, the entire root system per plant was stained according to the method of Philips and Hayman (1970) using acid lactic. Using mycorrhizal and non-mycorrhizal roots obtained for each level of dilutions and for the five repetitions, the number of propagules present in soil was evaluated with the help of the table of Cochran (1950). Furthermore, the mycorrhizal potential of soil was calculated for the following treatments: IS+SS, IM+SS, IS+NS, IM+NS and NI+NS.

2.7. Statistical analysis

Before analyses, all data were checked for normality and homogeneity of variance. We used analysis of variance (ANOVA) by a general linear model (GLM) to examine the effects of inoculation with AMF, EMF individually or in mixtures on the amelioration of the colonization levels and the growth of cork oak. The means were compared using Tukey's HSD test ($P < 0.05$). The Pearson correlation test is performed using an analysis of variance (ANOVA) and their plots were drawn using the package {ggplot2} (Chang, 2013). Statistical analyses and models were carried out using the software R.

3. Results

3.1. Plant growth

The inoculation by the ectomycorrhizal fungi (Ectovit), the arbuscular mycorrhizal fungi (Symbivit) and the dual inoculation of both had a significant impact on most of the measured cork oak plant growth parameters (Table 1). Overall, inoculated plants had better growth compared to the non-inoculated ones (i): height (F=88.08, P≤0.000), +73%, shoot fresh weight (F=169.75, P≤0.000), + 188%, root fresh weight (F=101.81, P≤0.000), + 147%, chlorophyll content (F=72.70, P 0.000), + 23%. (ii): height +14%, shoot fresh weight +35 %, root fresh weight + 38%, chlorophyll content + 10%. (iii): height +32%, shoot fresh weight + 111%, root fresh weight 86%, chlorophyll content + 35%. No significant difference (F=2.13, P≤0.109) was observed for the ratio root/shoot between the inoculated plants and the non-inoculated.

The treatment IE had the greater growth in comparison with IM and IS treatments. Significant differences were recorded. (i): IE compared to treatment IM +30% height + 36% shoot fresh weight, + 33% root fresh weight, (ii): IM compared to treatment IS, + 15% height, + 56% shoot fresh weight, + 34%, root fresh weight, +22% chlorophyll content. (iii): IE compared to treatment IS + 51% height, + 113% shoot fresh weight, + 79%, root fresh weight, + 12% chlorophyll content.

Furthermore, the effect substrate also showed significant differences on the shoot fresh weight (F=17.62, P≤0.000), the root fresh weight (F=28.36, P≤0.000), the ratio: root/shoot (F=16.56, P≤0.000) and the chlorophyll content (F=29.97, P 0.000). The interaction 'inoculation x soil treatment' was significant for the shoot fresh weight (F=9.15, P≤0.000), the root fresh weight (F=6.48, P≤0.001), the ratio: root/shoot (F=6.45, P≤0.001) and the chlorophyll content (F=5.02, P≤0.004).

3.2. Mycorrhizal root colonization

The mycorrhizal root colonization was observed in plants, both inoculated and non-inoculated with a commercial inoculum: IE, IS and IM (Table 1). However, the overall percentage of mycorrhizal colonization was significantly higher in the inoculated plants compared to the non-inoculated ones: (i): ectomycorrhizal root colonization (F=135.39, P≤0.000), + 279%, arbuscular mycorrhizal root colonization (F=69.18, P≤0.000), + 100%. (ii): ectomycorrhizal root colonization, + 141%, arbuscular mycorrhizal root colonization, + 300%. (iii): ectomycorrhizal root colonization, + 217 %, arbuscular mycorrhizal root colonization, +400 %.

Table 1. Height (H), shoot fresh weight (SFW), root fresh weight (RFW), ratio: root fresh weight/shoot fresh weight and chlorophyll content (CHC), ectomycorrhizal root colonization (EM%) and arbuscular mycorrhizal root colonization (AM%) of cork oak plants inoculated and non-inoculated with a commercial inocula EMF (IE), AMF (IS) and mixture of both inocula (EMF + AMF) (IM) grown on sterile substrate (SS) and non-sterile substrate (NS)

Parameters	Treatments								Significance 'Inoculation x soil treatment'
	Inoculation					Soil treatment			
	IE	IS	IM	NI	Significance	SS	NS	Significance	
H (cm plant-¹)	51.7 A	34.1 C	39.50 B	29.8 D	P<0.05	38.4 A	39.20 A	P=0.421	P=0.065 NS
SFW (g plant-¹)	4.9 A	2.3 C	3.6 B	1.7 D	P<0.05	3.3 A	2.9 B	P=0.000	P=0.000 ***
RFW (g plant-¹)	5.2 A	2.9 C	3.9 B	2.1 D	P<0.05	3.2 B	3.9 A	P=0.000	P=0.001***
Ratio root/shoot	1.1 A	1.3 A	1.1 A	1.5 A	P=0.109	1 B	1.5 A	P=0.000	P=0.001 ***
CHC(SPAD)	40.2 B 56.2	35.8 C 35.8	43.9 A 47.0	32.3 D	P<0.05	36.4 B	39.7 A	P=0.000	P=0.004 **
EM (%)	A 0.2 B	C	B	14.8 D	P=0.000	46.8 A	29.8 B	P=0.000	P=0.000***
AM (%)		0.4 A	0.5 A	0.1 C	P=0.000	0.3 A	0.3 B	P=0.021	P=0.001***

The means followed by the same letter within a column are not significantly different at $P < 0.05$ using Tukey's HSD test. *P <0.05, **P <0.01 and ***P <0.001**,** NS: no significance.

The ectomycorrhizal root colonization of cork oak plants inoculated with IE was significantly higher (56.2%) compared to IM and IS treatments (47% and 35.8%) presenting a gain of + 19% and + 56% respectively. The inoculation with IM also improved the EM root colonization which was greater by + 31% and + 19% compared to the previous treatments IS and IE. Conversely, no significant difference was detected for the

AM root colonization in the inoculated plants with the IM and IS treatments. The interaction 'inoculation x soil treatment' was significant (Table 1).

3.3. Mycorrhizogenic potential of soil

The number of infective propagules of indigenous AMF (MPN) in the soil before inoculation was 900 per 50 g of soil. The treatments IM and IS grown on the non-sterile substrate had a higher number of propagules: 2800 and 1800 respectively. In addition, the treatments IM and IS grown on the sterile substrate have shown MPN varied between 1500 and 1300 (Fig.1).

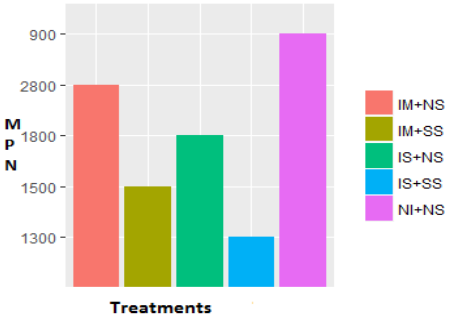

Figure 1. Number of propagules per kg of soil depending on the inoculum and the substrate used. (IS): arbuscular mycorrhizal inoculum, (IM): mixture inocula of ectomycorrhizal fungi and arbuscular mycorrhizal fungi, (SS): sterile substrate, (NS): non-sterile substrate

3.4. Correlations between mycorrhization and growth parameters

The relationships between the mycorrhization and the growth parameters revealed positive correlations between the ectomycorrhizal colonization rate (EM%) and the height (H), the fresh aerial biomass (SFW), the fresh root biomass (RFW), and the chlorophyll content (CHC), respectively 0.68, 0.66 and 0.80 and 0.76. Arbuscular mycorrhizal (AM%) showed a positive correlations with RFW, CHC and EM% by the coefficients of 0.23, 0.53 and 0.40. The CHC has recorded positive correlations with H, SFW, RFW, EM%, respectively 0.50, 0.52, 0.62, 0.76 and 0.53. Finally, the ratio root/shoot (R/S) is negatively correlated with SFW by a coefficient of -0.48 (Fig. 2).

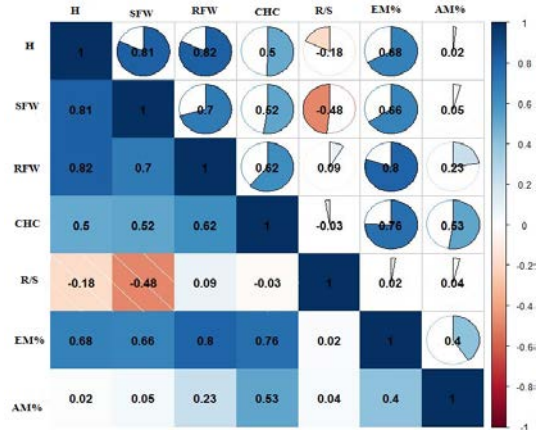

Figure 2. Pearson correlations between mycorrhization and growth parameters. Height (H), Shoot fresh weight (SFW), root fresh weight (RFW), ratio root/shoot (R/S), chlorophyll content (CHC) ectomycorrhizal root colonization (EM%) and arbuscular mycorrhizal (AM%). Numbers in the squares are correlation coefficients.

4. Discussion

The inoculation of cork oak plants with the arbuscular mycorrhizal fungi, ectomycorrhizal fungi and combination of these inoculum grown on both substrates (sterile and non-sterile) has significantly improved the mycorrhizal root colonization and the growth parameters. Whereas, the non-inoculated plants grown on the same substrates showed the low root colonization what has resulted in a low growth for all the measured parameters. Similarly, the number of propagules has been little compared to that obtained in the IM and IS treatments on the sterile substrate. The treatment IE recorded the highest root colonization levels, height and shoot and root fresh weight in comparison with the plants inoculated by IS and IM treatments. The results of this study are also in agreement with findings of Sebastiana *et al.* (2013) and Denis *et al.* (2015) who reported that the effect of inoculation with ectomycorrhizal fungi significantly increase the shoot and the root parts, the chlorophyll content and the performance of cork oak seedlings at nursery stage. The inoculation by the ectomycorrhizal fungi has also shown a positive effect on the growth of other forest trees as *Pinus tabulaeformis* Carr (Lu *et al*., 2016) and *Acacia mangnium* Willd (Diagnea *et al*., 2013). In the present experiment, the observed interaction between the substrate and the inoculation was significant indicating that the development of the seedlings is affected by these two factors. In parallel, similar studies revealed the significant effect between the substrate and the inoculation by ectomycorrhizal fungi on the growth of Pinus pinaster L. (Sousa *et al*., 2011) and *Picea abies* Karst. (Repac *et al*., 2015).

The treatment IS had less effect on the arbuscular mycorrhizal root colonization compared to the two previous treatments IM and IE, whereas it had a positive impact on the EMF root colonization. This may be due to the faster germination and growth of the arbuscular mycorrhizal propagules that can support hyphal growth in the direction of roots, or the quantity of existent propagules (Santos *et al*., 2001). The cork oak is a woody species, which has a strong symbiotic affinity to EMF at a more advanced stage of growth that would explain the low mycorrhizal intensity of the cork oak roots. Indeed, the eucalyptus forms AMF at the juvenile stage followed by EMF in adult age (Chen *et al*., 2000), Selosse *et al*. (2006) also reported that the AMF is generally the first to install and are joined by the EMF. The low AM colonization had also improved the growth parameters corroborating the results of other studies of *Salix repens* L. (Van der Heijden, 2001, Van der Heijden and Kuyper, 2003).

The dual inoculation (IM) had significantly improved the ectomycorrhizal colonization compared to treatments IE and IS. These observations suggest a succession in this dual system, which may be due to the rapid adaption of the AMF to primary infection within individual roots, and once the EMF is established the arbuscular mycorrhizal fungi might spread rapidly to new initials root, perhaps enabling the subsequent entrance of the AMF. The significant effect of the double inoculation on the root colonization may also be due to the competition and the interaction between the different inocula of the EMF and the AMF. This result is in the same line with the study of

(Chilvers *et al*., 1987) which proved that the differences observed in percent of roots colonized by AMF and EMF may be due to the competition between both of them. The influence of the dual inoculation on the arbuscular mycorrhizal root colonization is confirmed by the study of Michelsen *et al*. (1998) who concluded that the AMF and EMF were exploring separate pools of soil nutrients, thus suggesting a better resource exploration by tripartite symbiosis (plant, arbuscular and ectomycorrhizal fungi). Gagné *et al*. (2005) and Aggangan et al. (2010) have also demonstrated that the dual inoculation had enhanced the growth parameters of the plant species of the genus *Acacia* and *Eucalyptus*.

The low number of propagules was recorded in the non-inoculated plants while the highest number was obtained in the inoculated plants grown on the non-sterile substrate. The best mycorrhizal partners of cork oak are the ectomycorrhizae. However, despite the little presence of AMF in the roots of cork oak, the MPN is enriched by the synergistic intake of the dual inocula of (AMF+EMF) in the non-sterile substrate, even exceeding the intake of arbuscular mycorrhizae. This could be due to the composition of mycorrhizal fungi species of the inocula, Ectovit, Symbivit and their interactions with soil microflora. Dhillion and Gardsjord (2004) support this result while emphasizing that the specific composition, the productivity and the biodiversity of the epigeal flora were influenced by the composition and the specific richness of mycorrhizal communities. In the face of the AMF, which is not very specific to the host plant (Chagnon *et al*., 2012), the relative absence of specificity not only enables the fungi symbiotes to infect different plant species but also to form mycelial networks between plants. This could explain the increase in the number of propagules of plants inoculated by the AMF. Sanon *et al*. (2006) and Bilgo *et al*. (2011) have also shown that the AMF *Glomus intraradices* inoculated in sterilized soil could be maintained in a non-sterilized one (in the presence of native flora) and continue its beneficial activity to the host plant.

The positive significant relationships found between plant productivity and EMF infection parameter may be a consequence of the functional diversity of EMF (Hazard *et al*., 2017). This can be explained by the difference in mycorrhizal response within the same plant species for the same fungal strain. Indeed, it is difficult to determine the similarities, differences and the variance in the behavior of different plant species and even cultivars with the respect to the mycorrhizal symbiosis (Estaún *et al*., 2010).

5. Conclusion

This study showed that inoculation by the arbuscular mycorrhizae, the ectomycorrhizae and the dual inoculation by these two fungi, had positive effects on root colonization and growth of cork oak plants in the nursery. However, the mixture of inocula had significantly increased the mycorrhizal soil potential, enhanced by the interactions between the different populations of native mycorrhizae and the exogenous inputs of ectomycorrhizae and arbuscular mycorrhizae.

This research highlights that the controlled inoculation based on commercial mycorrhizal fungi can be a biotechnological technique in the improvement of the quality of seedling stock of *Q. Suber* and its performance after out-planting in the forests.

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Arsenic Induced Oxidative Perturbations in Freshwater Air Breathing Fish *Clarias batrachus*: *In Vivo* Study

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Abstract

Arsenic is ubiquitously prevalent metalloid contaminant released in the aquatic environment as a consequence of geogenic and anthropogenic processes. Bihar is emerging as hot spot of Arsenic toxicity in India and needs immediate environmental concern. In the present study, freshwater walking catfish *Clarias batrachus* (Linnaeus,1758)) was used as an experimental model to investigate oxidative stress related enzyme activities in serum. Fish were procured from Phulwarisharif Fish farm, Patna, Bihar, India, acclimated in the laboratory under ideal physiochemical condition for 15 days, and fed *ad libitum*. LC_{50} of fish for Arsenic trioxide was determined by probit regression analysis and confirmed by pilot test. The fish were exposed to 3.66 mg/L, 5.5 mg/L and 11.0 mg/L of arsenic trioxide for one, two and four week respectively. After schedule exposure, the fish serum of different groups was analysed for lipid peroxidation (LPO), superoxide dismutase (SOD), Catalase (CAT) and reduced Glutathione (GSH). Significant increase in the level of LPO was observed at all levels of arsenic exposure. A dose dependent notable decrease was observed in the activities of SOD, GSH and CAT. Statistical analysis showed a significant positive correlation between elevated levels of LPO and reduced GSH, SOD and CAT of the serum. The results of the present study indicate that sub-acute (two weeks) or chronic exposure (four weeks) of even a sub-lethal dose of arsenic trioxide induces significant oxidative stress in freshwater fishes. The assessment of oxidative parameters may be used as a biomarker for the health status of fish as well as aquatic body.

Keywords: Arsenic trioxide, oxidative stress, *Clarias batrachus*, Lipid peroxidation (LPO) superoxide dismutase (SOD), Catalase (CAT), Reduced Glutathione (GSH).

1. Introduction

Freshwater ecosystem has been subjected to immense toxicants since last two decades with the advent of industrial technology and non-judicious extensive use of different synthetic chemicals like fertilizers, pesticides, insecticides, herbicides *etc.* and generated potential threat for the survival of the aquatic life (Mandour *et al*., 2012; Mashkoor *et al*., 2013; Ghaffar *et al*., 2014; Witeska *et al*., 2014). It serves as major vector for pollutant dispersion acting as sink for environmental contaminants (Amundsen *et al.*, 2011). Undesirable changes are induced in the aquatic biota by these contaminants and ultimately affect the ecological balance (Witeska *et al*., 2014).

Heavy metals and metalloids are a consistent source of pollution of natural aquatic bodies. They contaminate fish via their general body surface, gills or digestive tract and induce disturbances in various physiological and biochemical mechanisms of fish, including alteration in carbohydrate, protein and fat metabolism, reducing cellular adaptive immunity and inducing histopathological anomalies in their vital organs (Abdel-Rahman *et al*., 2011; Mandour *et al*., 2012). Fishes are the best sentinels for determining the health status of an aquatic ecosystem as well as its potential impact on human health. Identification, monitoring and management of these pollutants are crucial in order to minimize their adverse effect on aquatic ecosystem.

Arsenic is a ubiquitous metalloid "pnictides" located on group V of the periodic table (Smith, 1973). It is introduced in the environment from both natural and anthropogenic sources (Abdel Hameid, 2009; Baldissarelli *et al.*, 2012; Rahman and Hasegawa, 2012; Aruljothi *et al*., 2013). Natural resources of Arsenic include withering of rock, leaching, run off, volcanic and biological activities (Rehman and Hasegawa, 2012; Zhang *et al*., 2014). Anthropogenic sources include- extensive mining and geothermal activities, use of metallic arsenic for strengthening alloys as well as in the processing of glass, pigments, textiles, parts, metal adhesive wood preservatives and ammunition (Thompson *et al*., 2007). According to USEPA aquatic life criteria, the acceptable limit of total arsenic concentration is 340 µg/L for acute exposure and 150 µg/L for chronic exposure in freshwater. However, 1.5 – 3.8 mg As^{3+}/L of water has been considered safe for fish (NAS, 1977). The concentration of arsenic for effective weed control (13.6 mg/L) has been reported to be harmful to several fish species (NRCC, 1978; Sorensen *et al*., 1985).

Air breathing fishes can concentrate and metabolize water borne pollutants and heavy metals effectively via antioxidant defense system, thereby considered as an excellent subject for the study of various effects of contaminants present in water sample (Valco *et al.*,2005).

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They have been utilized as biomarker to indicate the existence of toxicant exposure and/or the impact towards the evaluation of molecules cellular to physiological level (Sabullah *et al.*, 2015). Since fishes occupy the apex of the aquatic food chain, they have been considered as suitable bio-indicator of heavy metal contamination. Arsenic is known to induce oxidative stress in different organism. It is linked with the generation of reactive oxygen species (ROS) and reactive nitrogen species (RNS) free radicals viz. hydrogen peroxide (H_2O_2) (Wang *et al*., 1996; Chen *et al*., 1998); hydroxyl radical (OH) species (Wang *et al*., 1996), nitric oxide and super oxide anion (O_2) (Barchowsky *et al*., 1999; Lynn *et al*., 2000), Dimethyl arsenic peroxyl radical ($[CH_3]_2ASOO^-$]) (Yamanaka *et al*., 1991, 1997 & 2001) and dimethyl arsenic radical.

The present research work has been designed to investigate the oxidative biomarkers in the blood sample of freshwater walking catfish *Clarias batrachus* (Linn.) exposed at sub-lethal level of arsenic through LPO induction, and assessment of glutathione dependent antioxidant defense enzymes.

2. Materials and Methods

2.1. Test Chemicals

Arsenic trioxide (AS_2O_3) CAS No.13629 was purchased from Nice Chemicals Private Limited, Cochin. Its commercial grade formulation was white, powdery and insoluble in water. Reduced Glutathione (GSH), L chloro, 2-4 dinitrobenzene (DNB), Hydrogen peroxide (H_2O_2), 5-5'dithio-bis-2 nitrobenzoic acid (DTNB) and Trichloro acetic Acid (TCA) were purchased from Sisco Research Laboratory, Mumbai, while lauryl sulphate, Glacial acetic acid, Thiobarbituric acid (TBA), Pyrogallol were purchased from Hi Media Laboratories Private Ltd., Mumbai, India.

2.2. Test animal

Freshwater walking catfish *Clarias batrachus Linn*. (Siluriformes: Clariidae), commonly known as 'Magur,' was selected as test animal. It is hardy and restraining to most of the adverse ecological condition and mild xenobiotic exposure. It has a fast growth rate & prolific breeding ability. A very high content of protein (20.80 gm/100 gm tissues) in its flesh (Singh *et al*.,2016) and high percentage of haemoglobin in its blood (Maheshwaran *et al*.,2008) have raised its nutritional and therapeutic value. It inhibits all forms of swampy, marshy and derelict water. Nearly 380 Fresh healthy *Clarias batrachus (Linn)* with almost same age group and relatedness of mean body length 18±2 cm and mean body weight 74±6 gm were procured from Phulwarisharif Fish farm, Patna, Bihar, India.

2.3. Fish Care & Maintenance

After procurement fishes were brought to the Aquatic Toxicology laboratory, Department of Zoology, Patna University, Patna, Bihar and were first disinfected with 0.1% $KMnO_4$ solution bath for 20 minutes. The fishes were then maintained in nineteen plexiglass aquaria of 76.2cm x 45.72 cm x 45.72 cm dimension with 30L of de-chlorinated bore-well water @ 20 fishes/aquarium. Any disease or wounded fish, showing abnormal behaviour were removed from the aquaria immediately. The fishes were acclimatized in the laboratory under normal physico-chemical parameter i.e. room temp 27-30^{o}c, pH 7.2-7.5 and dissolved O_2 content 6.0-6.5mg/l as per the protocol followed by APHA(2012). The optimal condition during acclimatization was maintained (Benette and Dooley, 1982). The fishes were fed alternately with minced pellets of suji & egg and goat liver once daily @3-4% of body weight. The water was changed periodically.

2.4. Determination of sub lethal concentration

The 48 hours and 96 hours LC_{50} of *Clarias batrachus* for AS_2O_3 was determined according to graphical interpolation method (Doudoroff *et al*., 1951) and probit regression analysis (Finney, 1971). Later, it was confirmed by pilot test.

For the determination of median tolerance limit (LC_{50}), a group of 10 fishes was kept in six glass aquaria of 30 L capacity. A group of 10 fishes was kept in a separate aquarium and treated as control. After range finding test, six consecutive concentrations of AS_2O_3 *i.e.* 25 mg/l, 50mg/L, 75mg/L, 100mg/L, 125mg/L and 150mg/L respectively were added separately in each aquarium to determine the 48 hours and 96 hours LC_{50}.The test water was removed and fresh solution of AS_2O_3 was added daily to remove faecal wastes and to maintain the concentration of the test solution throughout the experimental duration under ideal condition. The median value was determined by plotting the experimental data on semi-logarithmic coordinate paper with test concentration on logarithmic scale and the percentage of survival on the arithmetic scale. A straight line was drawn between two points which are above or below the 50% survival line. The point of intersection with 50% survival line was considered LC_{50}.The experiment was done in triplicate. In the present study, the 48 hours LC_{50} and 96 hours $LC_{50}$of AS_2O_3 for *Clarias batrachus* was found at 130mg/L and 110mg/L respectively. Nearly similar value of LC_{50} was also calculated by probit regression analysis using SPSS software.

2.5. Experimental design

In the present study, fishes were grouped into four-(i) Control group (without AS_2O_3 exposure), (ii) SL-I group (Fishes treated with 3.66mg/L of AS_2O_3 i.e. 1/30th of LC_{50}), (iii) SL-II group (fishes treated with 5.5mg/L i.e. 1/20th of LC_{50}) and (iv) SL-III (fishes treated with 11 mg/L of AS_2O_3 i. e. 1/ 10th of LC $_{50}$).The stock solution for considered doses was prepared in dilute acidic water (stock solution A- 3.36 mg/L of AS_2O_3 was dissolved in 996.33 ml of distilled water; stock solution B-5.5 mg/L of AS_2O_3 was dissolved in 994.50 ml of distilled water; stock solution C-11 mg/L of AS_2O_3 was dissolved in 989 ml of distilled water).

A batch of 90 healthy fishes was placed in nine separate plexi glass aquaria @ 10 fishes each, and exposed to three sub-lethal concentrations i. e. 3.66 mg/L, 5.5 mg/L and 11mg/L of AS_2O_3 for one, two and four week respectively. The toxicant solution in the text aquaria was replaced after interval of 24 hours with the fresh solution of the same concentration. Three parallel groups of 10 fishes each were also maintained in arsenic free water under similar physico-chemical condition and treated as control throughout the experimental tenure.

At the termination of schedule time exposure, both treated and control group of fishes were collected and

anaesthetized under MS222 @100 mg/L. In each case, blood was collected in a heparinize glass syringe by puncturing caudal vein, allowed to clot and then centrifuged @3000rpm for 20 minutes. Clear supernatant was collected in fresh RIA tube, labeled properly and stored at 4^0c for biochemical analysis. The entire experiment was done in triplicate at three points of time.

2.6. Biochemical estimation:

2.6.1. Lipid peroxidation (LPO):

The level of LPO in serum was estimated by the standard method of Ohkawa *et al*. (1979) with thio-barbituric acid (TBA) and colour reaction for malonaldehyde (MDA). 1 ml of test serum sample was taken with equal volume of 0.67%w/v TBA, vertoxed for two minutes and kept on boiling water bath for 20 minutes, cooled and diluted with 1ml of TDW. The pink colour absorbance was read against blank at 532 nm in Spectronic 20 Bausch & Lamb spectro-photometer. The concentration of MDA was read from a standard calibration curve plotted using 1, 1, 3, 3'tetra methoxy propane (Sigma Aldrich Co. St Louis. USA) as standard, and the results were expressed as µmole of MDA/ml.

2.6.2. Superoxide dismutase (SOD):

The estimation of SOD was done by standard method (Markland & Markland, 1994). The method utilized the inhibition of auto oxidation of pyrogallol by SOD. The results were expressed in Unit/ml. 1 unit of SOD was considered as the amount of enzyme required to cause 50% inhibition of pyrogallol auto oxidation /3 ml of assay mixture.

2.6.3. Catalase (CAT):

The catalase activity was measured as per standard method of Sinha (1972) and Giri *et al*. (1996). Dichromate is reduced to chromic acetate with the formation of per-chromic acid as an unstable intermediate complex when treated with H_2O_2 in the presence of acetic acid. The chromic acid is measured colorimetrically at 620 nm. CAT split H_2O_2 at different time intervals by the addition of a dichromatic acetic acid mixture and remaining H_2O_2 is determined colorimetrically. The results were expressed as µmole of H_2O_2 utilized/ml.

2.7. Reduced Glutathione (GSH):

The estimation of glutathione was done by standard method (Ellman, 1959 modified by Beutler *et al*., 1963). The sulfhydryl group of GSH reacted with Ellman reagent (5-5'dithio-bis-2 nitro-benzoic acid (DTNB) and produced a yellow coloured 5-thio, 2-Nitro benzoic acid (TNB) which was measured on spectro-photometer at 412 nm. The results obtained were expressed as glutathione mg/dl in blood.

2.8. Statistical analysis:

For the estimation of each oxidative parameter six (n=6) observations at random were taken and the arithmetic mean in each case was calculated and subjected to statistical analysis. The standard deviation, standard error of mean and one way standard analysis of variance (ANOVA) was applied to determine the significance difference between the different experimental mean. Paired 't'-test was applied between control and different arsenic treated groups. A value at $p<0.05$ and <0.01 was considered statistically significant. All the statistical analysis was done on SPSS 17.0 version.

3. Results

The present research work was undertaken to assess the arsenic induced oxidative stress and related anomalies in the serum of fresh water air breathing fish *Clarias batrachus (Linn.*).To address our hypothesis, the effect of AS_2O_3 on the four oxidative parameters i.e. LPO, SOD, CAT and GSH was studied at three different concentrations of AS_2O_3 for one, two and four week respectively. The inter-relationship of four different oxidative parameters and their fluctuations in response to arsenic exposure was significantly analyzed.

3.1. Effect of AS_2O_3 on serum LPO:

The data of serum LPO in all the four groups are presented in text table 1 and also illustrated with the help of logarithmic Figure 1A, 2A and 3A. It was observed that the level of serum LPO uniformly mounted in all three test groups at all exposure level.

Table 1. LPO (Mean ± SEM) of control and treated *C. batrachus* exposed to three sub-lethal concentrations of As_2O_3 for one week, two weeks & four weeks

Group	LPO level (µmole/ml) Period of Exposure		
	1 week (Mean±SEM)	2 week (Mean±SEM)	4 week (Mean±SEM)
Control	1.435±0.232	1.435±0. 232	1.435±0. 232
SL-I	1.920±0.185* (+33.79%)	2.363±0.340* (+64.67%)	2.266±0.228* (+57.90%)
SL-II	2.784±0.392* (+94.07%)	3.151±0.236** (+119.587%)	6.820±0.290** (+375.26%)
SL-III	2.848±0.47** (+98.47%)	2.546±0.06* (+77.42%)	4.500±0.228* (+213.58%)

Values are expressed in mean ± SEM of six replicate (n=6) in each group. Paired't'-test was applied between control and different arsenic treated groups. Significant response: * $p<0.05$, ** $p<0.01$. One way 'ANOVA test' was done to observe overall variation in LPO. Calculated F-value is 33.341 while table F-value is 2.18. Values in Parenthesis are % increase over control.

3.2. Effect of AS_2O_3 on SOD

A significant decline in serum SOD was marked at higher duration of all the three exposure level. The data of serum SOD in all the four groups at all the level of exposure have been shown in table 2 and illustrated in figure 1B, 2B and 3B.

Table 2. SOD (Mean ± SEM) of control and treated *C. batrachus (Linn.)* exposed to three sub-lethal concentrations of As_2O_3 for one week, two weeks & four weeks respectively.

Group	Serum SOD level (unit/ml) Period of Exposure		
	1 week (Mean±SEM	2 week (Mean±SEM)	4 week (Mean±SEM)
Control	1.379±0.187	1.379±0.187	1.379±0.187
SL-I	1.242±0.090* (–9.93%)	1.387±0.060 (+0.58%)	0.942±0.16** (–31.83%)
SL-II	1.260±0.190* (–8.62%)	0.925±0.11** (–32.92%)	0.856±0.140* (-37.93%)
SL-III	1.280±0.233* (–7.17%)	0.903±0.15* (–34.51%)	0.548±0.023** (–60.26%)

Values are expressed in mean ± SEM of six replicate (n=6) in each group. Paired't'-test was applied between control and different arsenic treated groups. Significant response: * $p<0.05$, ** $p<0.01$, NS= Non- significant. One way 'ANOVA test' was done to observe overall variation in serum SOD. Calculated F-value is 15.777 while table F-value is 2.18. Values in Parenthesis are % increase (+) or decrease (–) over control.

3.3. Effect of AS_2O_3 on CAT

Text table 3 represents the concentration of serum catalase in control and all the three experimental groups. Initially serum CAT showed non-significant induction, but in all other exposure level significant reduction were marked, as illustrated in figures 1C, 2C and 3C.

Table 3. CAT (Mean ± SEM) of control and treated *C. batrachus (Linn.)* exposed to three sub-lethal concentrations of As_2O_3 for one, two and four week respectively.

Group	Serum CAT level (µmole/ml) Period of Exposure		
	1 week (Mean±SEM)	2 weeks (Mean±SEM)	4 weeks (Mean±SEM)
Control	135.56±2.47	135.56±2.47	135.56±2.47
SL-I	140±2.60^{NS} (+3.27)	110.65±1.85* (–18.37%)	92.65±2.569** (–31.65)
SL-II	115.65±8.86* (–14.68%)	82.56±7.25** (–39.09%)	65.75±2.02** (–51.49%)
SL-III	86.25±9.55** (–36.375%)	55.92±7.86** (–58.75%)	28.50±5.2** (–78.97%)

Values are expressed in mean ± SEM of six replicate (n=6) in each group. Paired‘t’-test was applied between control and different arsenic treated groups. Significant response: * p<0.05; ** p<0.01, NS= Non- significant. One way ‘ANOVA test’ was done to observe overall variation in serum CAT. Calculated F-value is 33.341while table F-value is 2.18. Values in Parenthesis represents percentage increase (+) or decrease (–) over control.

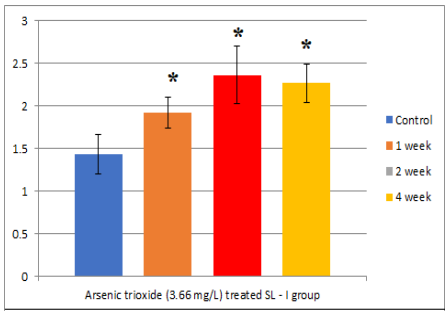
3.4. Effect of AS_2O_3 on GSH:

Text table 4 represents the concentration of blood GSH in control and all the three experimental groups. As it is clearly depicted in figures 1D, 2D and 3D, GSH, an important detoxifying enzyme, significantly decreased with increasing time of AS_2O_3 exposure against control.

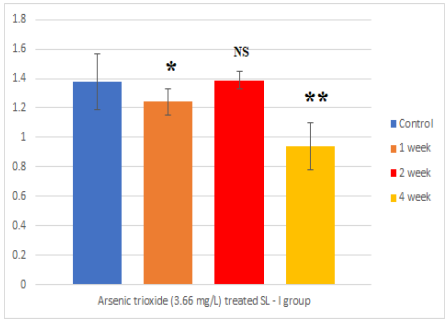
Table 4. GSH (mg/dl) of control and treated *C.batrachus (Linn.)* exposed to three sub-lethal concentrations of As_2O_3 for one, two & four week respectively.

Group	Blood GSH level (mg/dl) Period of Exposure		
	1 week (Mean±SD)	2 weeks (Mean±SD)	4 weeks (Mean±SD)
Control	57.409±2.21	57.409±2.21	57.409±2.21
SL-I	38.201±1.236* (–33.45%)	35.449±3.81* (–38.25%)	33.992±2.24* (–40.78%)
SL-II	33.411±1.92* (–41.80%)	24.819±3.10** (–56.76%)	17.163±2.30** (–70.10%)
SL-III	31.337±1.960* (–45.41%)	20.373±1.416** (–64.51%)	13.502±2.11** (–76.48%)

Values are expressed in mean ± SEM of six replicate (n=6) in each group. Paired‘t’-test was applied between control and different arsenic treated groups. Significant response: * p<0.05, ** p<0.01, NS= Non- significant. One way ‘ANOVA test’ was done to observe overall variation in blood GSH. Calculated F-value is 55.138 while table F-value is 2.18. Values in Parenthesis represented % decrease (–) in blood GSH over control.



(A)



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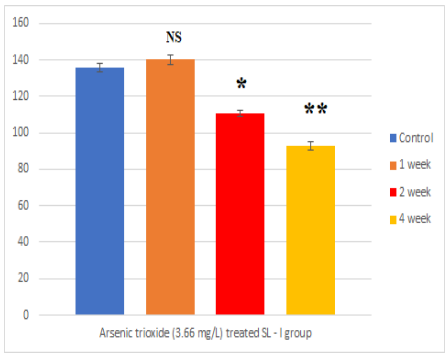

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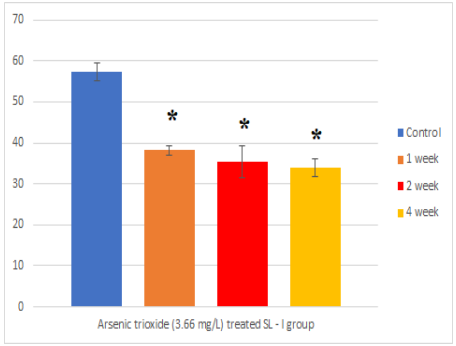
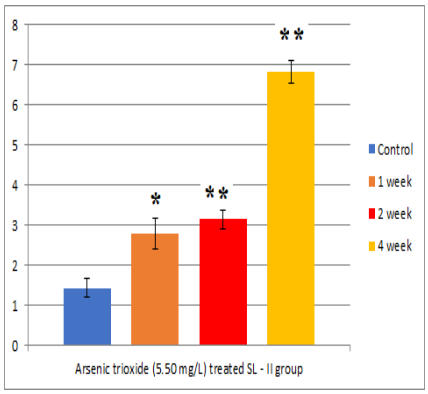
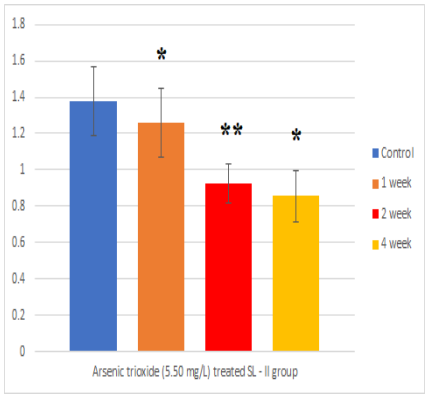


(D)

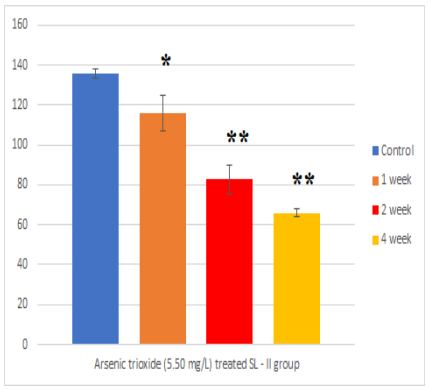
Figure 1. (SL – I group): Time dependent level of serum Lipid peroxidation-LPO (A), serum superoxide dismutase- SOD (B), Serum Catalase – CAT (C) and reduced Glutathione-GSH (D) in *Clarias batrachus* after exposure to 3.66 mg/L Arsenic trioxide. The results are expressed as mean ± SEM of six replicates (n = 6). * indicates significant differences at $p < 0.05$ and ** indicates significant difference at $p < 0.01$.



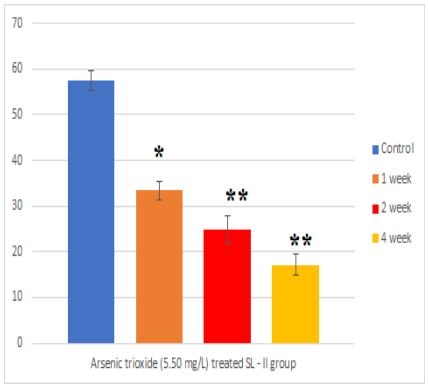
(A)



(B)

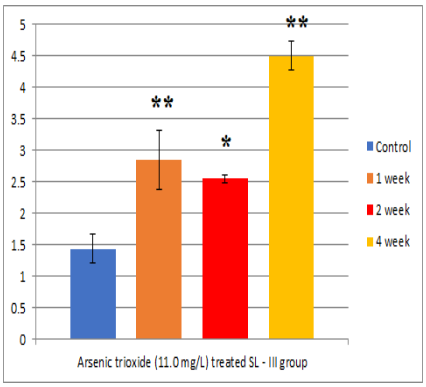


(c)

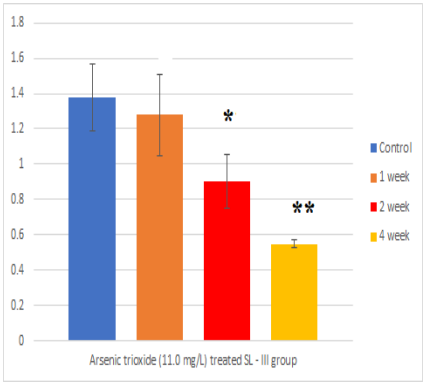


(D)

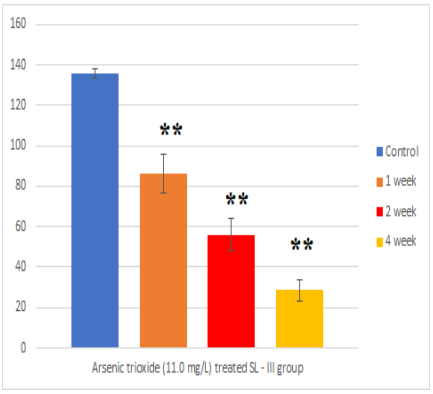
Figure 2. (SL – II group): Time dependent level of serum Lipid peroxidation-LPO (A), serum superoxide dismutase- SOD (B), Serum Catalase – CAT (C) and reduced Glutathione-GSH (D) in *Clarias batrachus* after exposure to 5.5 mg/L Arsenic trioxide. The results are expressed as mean ± SEM of six replicates (n = 6). * indicates significant differences at $p < 0.05$, and ** indicates significant difference at $p < 0.01$. NS = Not significant.



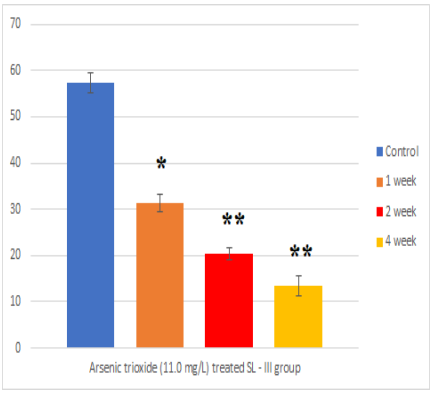
(A)



(B)



(C)



(D)

Figure 3. (SL – III group): Time dependent level of serum Lipid peroxidation-LPO (A), serum superoxide dismutase- SOD (B), Serum Catalase – CAT (C) and reduced Glutathione-GSH (D) in *Clarias batrachus* after exposure to 11.0 mg/L Arsenic trioxide. The results are expressed as mean ± SEM of six replicates (n = 6). * indicates significant differences at $p < 0.05$, and ** indicates significant difference at $p < 0.01$.

4. Discussion

In the present investigation, walking cat fish were exposed to 3.66 mg/L ($1/30^{th}$ of LC_{50}), 5.5 mg/L ($1/20^{th}$ of LC_{50}) and 11 mg/L ($1/10^{th}$ of LC_{50}) of As_2O_3 for one, two and four weeks respectively, and corresponding change in the oxidative parameters was studied. Three concentrations of AS_2O_3 were selected since they were below the lethal range with no mortality till 60 days, and were within the limit of USEPA aquatic life criteria. Similar sub-lethal range of AS_2O_3 has also been considered by several authors viz.3.8 mg/L *i.e.*1/20 LC_{50} and 7.6mg/L *i. e.*1/10LC_{50} (Roy & Bhattacharya, 2006) 42.42µM *i. e.* 1/10 of LC_{50} (Ghosh *et al.* 2006; 2007) and 35 µM *i. e.* 1/10 of LC_{50} (Roy *et al*., 2006).). The reason behind the consideration of duration of exposure in this experiment for one, two and four weeks, is that four weeks was found to be sufficient to reflect the effect of the toxicants and at the same time triggered the up-regulation of the detoxification system in fish (Datta et al.,2007). Review of literature suggests that pathway of xenobiotic metabolism systems in fish is analogous to higher vertebrates and efficient in eliminating a multitude of xenobiotics (Chatterjee and Bhattacharya, 1984; Sarkar,1997 ;Bhattacharya *et al*. 2007).

The reactive oxygen species (ROS) generation is the principal toxico-kinetics of arsenic (Farombi *et al*., 2007; Kitchin, 2001; Flora *et al*., 2005). As (III) exerts its toxic action either directly by attacking –SH group of an enzyme and thereby inhibiting enzyme action or indirectly through generation of ROS (Chen *et al*. 2001). The production of ROS is a normal aspect of cellular metabolism, but increased production of ROS leads to oxidative stress. Enhancement of intracellular ROS modulates the occurrence of cell damage via initiation and propagation of LPO (Gutteridge, 1995).

Lipid peroxidation refers to the oxidative degeneration of lipid. It is the process by which free radicals steal electron from the lipid in cell membrane resulting in considerable cell damage. It includes three basic steps: initiation, propagation and termination. Peroxidation of lipid can disturb the assembly of membrane causing changes in fluidity and permeability, alteration of ion transport and inhibition of metabolic processes. Mitochondrial damage through lipid peroxidation, directs further ROS generation and enhances the extent of oxidative stress (Brazilai and Yamamoto 2004; Varanka, 2004; Patra *et al*, 2011). Peroxidative damage due to AS (III) accumulation has been reported in *Anabas testudineus* (Roy *et al*., 2004), *Channa punctatus* & *Clarias batrachus* (Bhattacharya & Bhattacharya, 2005), *Tilapia mossambica* (Sounderarajan *et al.* 2009). Oxidative stress in fish results in free radicals generation, a reduction in antioxidant protection and failure to repair oxidative damage. In the present study, at lower sub-lethal exposure (SL-I group), LPO showed a significant increase ($P<0.05$) of 64.67% and 57.90% over the control within two week and four week duration. At median sub-lethal exposure (SL-II group), it showed a significant ($P<0.01$) increase of 375.26% over control. At higher sub-lethal concentration (SL-III Group), a significant ($P<0.05$) increase of 98.47%, 77.42% ($P<0.01$) and 213.58% ($P<0.01$) respectively over control has been observed at one, two and four week duration respectively. Similar kind of LPO inductions were found in zebra fish brain due to low dose of arsenic trioxide (Sarkar *et al.,* 2014). The consistent significant increase in serum LPO with the increasing concentration of As_2O_3 at sub acute and chronic exposure level in the present study can be correlated with increased ROS & RNS free radicals generation over time that completely disrupted the anti oxidative system in *Clarias batrachus.* Free radicals may be generated by the oxidation of carbohydrates, fats and proteins through both aerobic and non aerobic processes. ROS inactivates membrane transporters, damage DNA & transcriptional machinery (Pryor and Godber,1991; Vendemiale *et al.,*1999) and initiate a chain reaction that peroxidizes poly unsaturated fatty acids, leading to increased production of LPO (Cand and Verdetti, 1989; Rikans *et al.,*1997).

Antioxidant enzymatic defenses like SOD, CAT, GPX (Glutathione peroxidase) and GST (Glutathione S-transferase) play a massive role in protecting the tissue from oxidative damage (Valvanidis *et al*, 2006). Superoxide dismutase is an important endogenous antioxidant enzyme that act as first line of defense against ROS, catalyses dismutation of the superoxide anion & scavenges superoxide radicals & generates H_2O_2 (Winston & Digiulio, 1991; Gunney *et al*, 2009). Catalase and GPX are both involved in H_2O_2 removal in peroxisomes and cytosol by coupling its reduction to H_2O with oxidation of GSH. The activation of SOD requires concomitant activities of cytosolic GPX and/or catalase activity to protect the cell from oxidative stress (Halliwell and Gutteridge, 1999). In the present study, in SL-I Group SOD initially declines by 9.93% over control after one week exposure but then at 2 week exposure it elevates up to nearly normal value. At 4 week exposure, it again declines by 31.83% over the control. Similarly, in SL-II Group, it showed a consistent decline of 8.62%, 32.92% and 37.93% over the control at three exposure levels. At higher sub-lethal concentration of As_2O_3, a sharp decline of 34.51% and 60.26% over control was recorded at 2 week and 4 week exposure levels. In the present study, a slight elevation in SOD in SL-I group at sub acute exposure level marks the simultaneous activation of antioxidant system in fish, but huge accumulation of free radicals due to constant exposure of arsenic render the level of SOD and various enzymes of antioxidant system declining consistently. Significant decrease in serum catalase was observed at all exposure levels except a non-significant increase of 3.275% over control in one week exposure of SL-I Group. At lower sub-lethal concentration of As_2O_3 (SL-I), a significant decline of 18.37% ($p<0.05$) and 31.65% ($p<0.01$) over control was marked at two week and four week test exposure, while at higher sub-lethal concentration of As_2O_3 (SL-III) a significant decline of 36.37% ($p<0.01$), 58.75% ($p<0.01$) and 79.97 % ($p<0.01$) over control was marked at one, two and four week duration, respectively.

GSH plays a significant role in management of arsenic induced oxidative stress (Valco *et al.,*2005). In the present study, blood GSH showed a significant decline at almost every exposure level when compared with control. At lower sub-lethal concentration of As_2O_3, blood GSH showed a marked reduction by 33.45%, 38.25% and 40.78% over the control in one, two and four week, respectively. While at higher sub-lethal concentration a sharp decline in GSH by 45.41% ($p<0.05$), 65.51%

($p<0.01$) and 76.48% ($p<0.01$) over control was marked in one, two and four week exposure respectively. A similar decrease in reduced GSH in zebra fish gills and livers exposed to Dimethoate (Ansari and Ansari,2014) and cardiac tissues in rats as a consequence of short term arsenic toxicity was reported (Muthumani,2013). Elevation in CAT and GSH activities is usually related with the increased SOD concentration to neutralize toxic H_2O_2 in peroxisomes. But decline in CAT and GSH level in the present study can be related with extra ordinary generation of free radicals and diminished SOD due to consistent sub lethal exposure of AS_2O_3.

Catalase is very pertinent in protecting the cell from toxic effects of H_2O_2 and radical oxygen species (Coban *et al*. 2007, Altikat *et al,* 2006). In the present study, the reduction in the activities of CAT is found to be directly linked with the concentration and duration of As_2O_3 exposure. A similar decrease in CAT activity in the tissues of living animal exposed to arsenic has been reported (Wang *et al.* 2006; Ventura-Lima *et al*. 2009). Our findings are in agreement with the results of Altikat *et al.* (2014), who reported similar decline in CAT activities in all the tissues of *Cyprinus carpio carpio*, exposed to 0.5 and 1.0mg/L of sodium arsenite. A significant decrease in the CAT activities in liver and ovary of zebra fish in response to 0.006 and 0.03 ppm AS_2O_3 exposure has been reported and claimed by the authors that reduced CAT activity may be associated with the diminished level of NADPH in fish (Sunaina *et al.*,2016). Superoxide radicals inhibit CAT enzyme activity (Kono and Fridovich, 1982). The decrease in CAT enzyme activity as a result of arsenic exposure might be due to depression in protein synthesis caused by the free radical damage (Humtose *et al*. 2007; Palaniappan and Vijayasundaram, 2008). However, our findings are in contrast to the earlier reports of increased CAT activity after arsenic exposure (Bhattacharya and Bhattacharya, 2007).

5. Conclusion

The contamination of natural aquatic system with heavy metals released from domestic, industrial and other anthropogenic activities has become a matter of serious concern over the last few decades (Vutukuru, 2005; Conacher *et al*., 1993; Velez and Montoro, 1998). Fishes being at the apex of the aquatic ecosystem food chain, get easily victimized to their adverse effects (Al-Yousuf and El- shaahwi, 1999; Farkas *et al*., 2002). The aquatic bodies in State of Bihar are facing severe arsenic contamination over the last few years (Chakraborty *et al.,* 2003).The toxic effects of nonlethal concentration of arsenic on the various oxidative indices have been demonstrated in the present study. As per National Academy of Sciences, US (NAS, 1977) the approved safe limit of arsenic for fish is 1.5 – 3.8 mg/L of water, but the findings of the present study revealed a significant perturbations in the oxidative parameters of freshwater air breathing fish *Clarias batrachus (Linnaeus*, 1758) even at much lower concentration of arsenic *i.e*. 3.66 mg As^{3+}/L, than the approved limit of NAS. Oxidative stress in Indian major carps (IMC) have been even reported at further lower concentration of arsenic *i.e.* 0.5 mg/L and 1.0 mg/L (Altikat *et al.*, 2014). Induction of oxidative stress through ROS generation is one of the principal toxico-kinetics of As_2O_3 in aquatic organism as marked by significant increase in serum LPO level in the test fish at all levels of arsenic exposure. A dose dependent reduction in antioxidant enzyme system, i.e. SOD, CAT and GSH, clearly marks their failure in repairing oxidative damage in fish generated by arsenic exposure. Hence, the assessment of oxidative parameters of fish may be considered as bio-indicators of heavy metal toxicity of aquatic body. Considering the deleterious impact of arsenic on the aquatic system and their implications on human health, it is highly recommended to monitor the level of arsenic in edible fishes in Bihar consistently. It will be equally important to work on mitigation strategies for the bio-conservation of these fishes.

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Compliance with Ethical Standards Findings:

The study was not funded by any grant funding body.

Conflict of Interest

There is no conflict of interest in the study as declared by both authors.

Ethical Approval

All applicable international, national and/or institutional guidelines for the care and use of animals were followed.

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Landmark-based Morphometric and Meristic Variations in Emperors (*Lethrinus*, Lethrinidae, Percoidei) from Three Areas around Sulawesi (Indonesia) with Different Levels of Destructive Fishing

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Abstract

This study analysed the variation in morphometric and meristic characteristics among fishes in the genus Lethrinus from three areas around Sulawesi (Indonesia) with different levels of destructive fishing: Makassar, South Sulawesi, high; Manado, North Sulawesi, medium; and Wakatobi, Southeast Sulawesi, low. The research was conducted from June-November 2019. Morphometric characters (21) and meristic characters (8) of L. erythropterus, L. semicintus, L. obsoletus, L. ornatus, and L. harak were measured (30 specimens/species/site). Morphometric characters were compared between areas using one-way analysis of variance (ANOVA) with post-hoc Tukey Test. Characteristic traits and similarities between species/areas were evaluated using Multivariate Discriminant Function Analysis (DFA) test and tree diagram (dendrogram) analysis. For the five lethrinid species studied there were statistically significant differences ($P<0.05$) in morphometric characters between the Makassar population and the populations in Manado and Wakatobi. Interestingly, meristic count variability was greater in lethrinids from Makassar and Manado compared to those from the Wakatobi marine protected area. Differences in destructive fishing level between areas are one factor influencing lethrinid morphometric and meristic characters, which can also be influenced by other environmental conditions.

Keywords: Sulawesi, morphometric, meristic, destructive fishing, Lethrinidae

1. Introduction

Emperors (Perciformes: Lethrinidae) are a group of high-value coral reef-associated fishes found in tropical and sub-tropical waters. Emperors can be found from the back-reef area, including shallow seagrass beds, to the reef crest and slopes to depths of a few hundred meters (Carpenter and Allen, 1989). There are approximately 29 species of emperors in the Family Lethrinidae, of which 86% have been reported from Indonesian waters (Carpenter and Randall, 2003; Lo Galbo *et al*, 2002). The Lethrinidae are further divided into two subfamilies: the Lethrininae (with a single genus: *Lethrinus*) and the Monotaxinae (genera *Gnathodentex*, *Monotaxis* and *Wattsia*).

The high volume of exports and lack of regulation on the trade in fishes of the genus *Lethrinus* have given rise to concerns regarding the condition of these populations. Furthermore, in addition to heavy demand from the export market, *Lethrinus* populations are threatened by the various destructive fishing methods used to catch these valuable fish (Briggs, 2003). Destructive fishing is prevalent in the seas around Sulawesi, but severity is not uniform between areas (Unsworth et al., 2018; Briggs (2003) categorised the risk level as high in the waters off Makassar in South Sulawesi, medium in the waters off Manado in North Sulawesi, and low in the waters of the Wakatobi Archipelago in Southeast Sulawesi. The past 44 years have also seen changes in the coral cover of Sulawesi reefs (Nurdin *et al*, 2016; Nurdin *et al*, 2015; Edinger *et al*, 1998).

Fishing pressure and destructive fishing along with phenomena associated with global warming are increasing the pressure on fish communities (Andrés *et al*, 2013). Fishes of the genus *Lethrinus* are protogynous hermaphrodites (Marriott *et al*, 2010; Motlagh *et al*, 2010), changing from female to male after more than five years, at around 33 cm total length (Wassef, 1991). In addition to the high fishing mortality of small size classes, global warming phenomena are detrimental to fitness, with impacts at the population level including imbalance in the sex ratio of *Lethrinus* populations. This imbalance can lead to the emergence of cryptic species which can affect the population structure in multi-species *Lethrinus* communities, with the potential to severely affect the condition and even the survival of these fisheries resources, including changes in morphological characters.

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At an intra-species level, however, change in morphological characteristics is not always controlled principally by genetic factors, but rather by changes in the surrounding environment (Clayton, 1981). Phenotypic plasticity is strategy employed by many fishes to enable them to adapt to their environment through physiological and behavioural modifications which can lead to or entrain morphological and reproductive changes and alter survival rates in order to reduce the impacts of environmental change. Such strategies do not necessarily result in genetic changes within the population, and such morphological differences between populations cannot be used as proof of genetic differences (Meyer, 1987; Stearns, 1983).

In the context of stock management for fishes such as the genus *Lethrinus*, the analysis of differences in morphometric and meristic characters can enable the grouping of individuals or populations (Burhanuddin, 2014). Quantitative morphometric studies are an important key to determining sex and species (Brzesky and Doyle, 1988), describing the patterns of morphological traits which can differentiate between populations or species (Ballesteros-Córdova *et al*, 2016; Firawati *et al*, 2017), in taxonomic classification and the estimation of phylogenetic relationships, and in identifying new species (Burhanuddin and Iwatsuki, 2003). Landmark-based morphometric methods are increasingly used in the identification, discrimination, and classification of fishery stocks, and can also indicate differences in growth and maturation rates because body form is a product of ontogeny (Hayward and Margraf, 1987; Roby *et al*, 1991; Cadrin, 2000). These considerations prompted this research with the aim of analysing the biodiversity and population structure of *Lethrinus* species in waters around Sulawesi using an approach based on morphometric characters. The results of this research will contribute to the scientific basis for forming policies and outlining effective strategies to manage and conserve *Lethrinus* communities.

2. Materials and Methods

This research was carried out from June to November 2019 in three sea areas around Sulawesi, Indonesia: the waters of Manado City in North Sulawesi Utara; the waters off Makassar City in South Sulawesi; and the waters of the Wakatobi District (Wakatobi Archipelago) in Southeast Sulawesi (Figure 1). Samples were collected and then analysed at the Marine Biology Laboratory, Faculty of Marine Science and Fisheries, Universitas Hasanuddin, in Makassar.

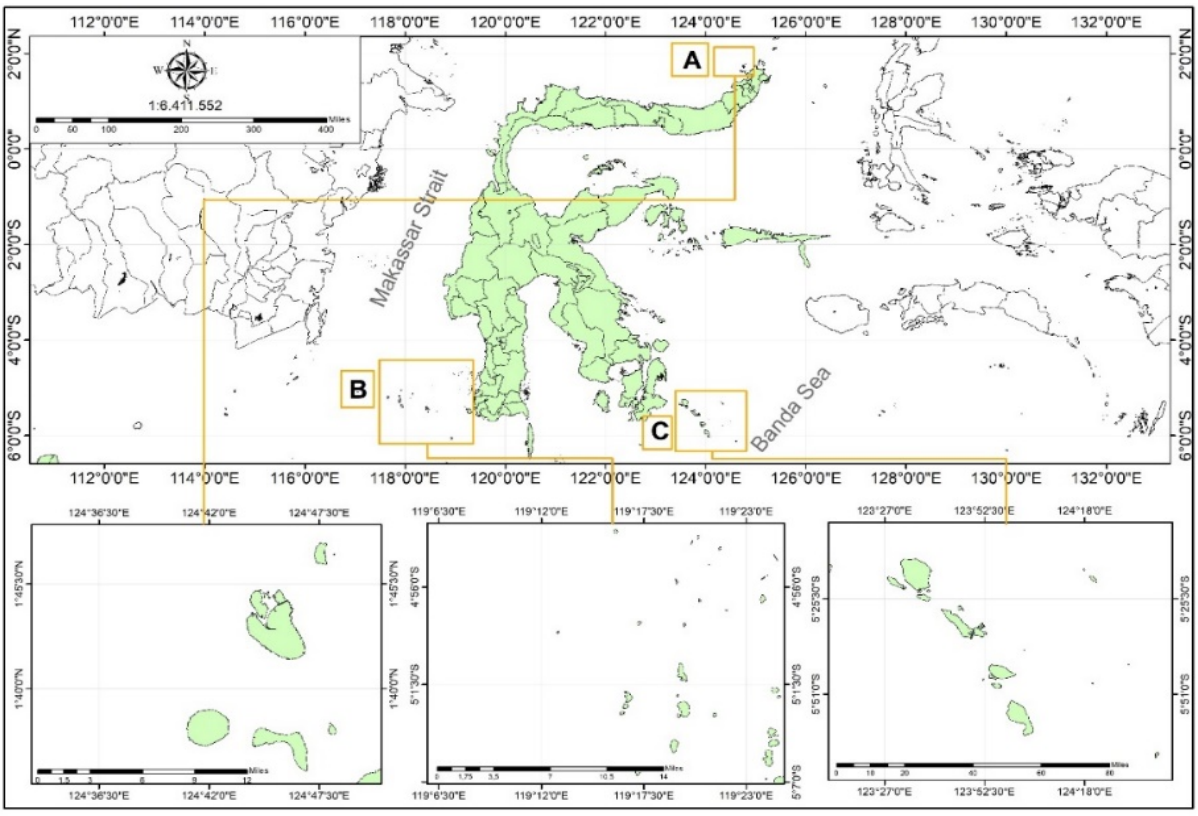

Figure 1. Sampling locations for Lethrinid fishes within Sulawesi waters: **(A)** Manado, North Sulawesi; **(B)** Makassar, South Sulawesi; **(C)** Wakatobi, Southeast Sulawesi.

The total number of fish sampled was 450 specimens, belonging to five species of the genus *Lethrinus: L. erythropterus, L. semicintus, L. obsoletus, L. ornatus, and L. harak.* The measurement of morphometric and meristic characters mostly followed Brzesky and Doyle (1988) and Hubbs and Lagler (1958) (Table 1, Figure 2), except for the number of gill-rakers (Burhanuddin *et al*, 2002). Measurements were made using digital callipers (WIPRO, WP-150B, precision 0.02 mm).

Table 1. Lethrinus morphometric characters measured, based on Brzesky and Doyle (1988).

Body Section	Code	Character Description
Head	A1	Posteriormost point of maxilla to origin of pelvic fin
	A2	Posteriormost point of maxilla to posteriormost point of eye
	A3	Posteriormost point of eye to origin of dorsal fin
	A4	Origin of pelvic fin to origin of dorsal fin
	A5	Posteriormost point of eye to origin of pelvic fin
	A6	Posteriormost point of maxilla to origin of dorsal fin
Anterior Body	B1	Origin of pelvic fin to origin of anal fin
	B3	Origin of dorsal fin to point between spinous and soft portions of dorsal fin
	B4	Origin of anal fin to point between spinous and soft portion of dorsal fin
	B5	Origin of dorsal fin to origin of anal fin
	B6	Origin of pelvic fin to point between spinous and soft portions of dorsal fin
Posterior Body	C1	Origin of anal fin to insertion of anal fin
	C3	Point between spinous and soft portions of dorsal fin to insertion of dorsal fin
	C4	Insertion of anal fin to insertion of dorsal fin
	C5	Point between spinous and soft portions of dorsal fin to insertion of anal fin
	C6	Origin of anal fin to insertion of anal fin
Tail	D1	Insertion of anal fin to anterior attachment of ventral membrane from caudal fin
	D3	Insertion of dorsal fin to anterior attachment of dorsal membrane from caudal fin
	D4	Anterior attachment of ventral membrane from caudal fin to anterior attachment of dorsal membrane from caudal fin
	D5	Insertion of dorsal fin to anterior attachment of ventral membrane from caudal fin
	D6	Insertion of anal fin to anterior attachment of dorsal membrane from caudal fin

The meristic characters counted were: (1) Dorsal fin-rays, (2) Anal fin-rays, (3) Pectoral fin-rays, (4) Pelvic fin-rays, (5) Lateral line scales, (6) Scales above lateral line, (7) Scales below lateral line, and (8) number of gill-rakers.

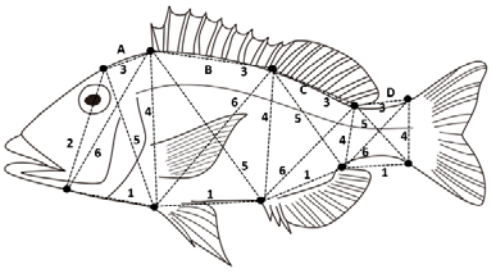

Figure 2. Morphometric truss for the genus Lethrinus, showing the location of the 10 anatomical points (landmarks) used and the morphometric distances measured for each individual (Brzesky and Doyle, 1988).

Before analysing the morphometric data, the data were standardised using the regression from Elliot *et al.* (1995). This model was used to remove the size component from the measurements of shape and homogenise the variation within the sample (Jolicoeur, 1963). The regression formula from Elliot *et al.* (1995) is:

$$M_s = M_o\left(\frac{Ls}{Lt}\right)^b$$

where M_s is the standardized measurement of the character, Mo is the original morphometric measurement (mm), *Ls* is the standard length of the fish, and *Lt* the mean of the standard length of all fish from all samples. The parameter *b* was estimated for each character from the observed data as the slope of the regression of log M on log L, using all specimens.

The standardised morphometric data for the 450 specimens was compared using One-Way analysis of variance (ANOVA) followed by post-hoc Tukey tests. Further analysis using Multivariate Discriminant Function Analysis (DFA) was used to identify characters specific (to a species or site), and resemblances between species or sites. These analyses were implemented in SPSS version 22.0. Principal Component Analysis (PCA) and tree (dendrogram) analysis were used to analyse the patterns of diversity between species at each site; these analyses were implemented in Minitab version 17.0.

3. Results

3.1. Morphometric Characters of Lethrinus spp.

The measurements of the morphometric characters of *Lethrinus* spp. species are given in Table 2. The results of the one-way analysis of variance followed by Tukey post-hoc tests (at 95% confidence level) are also shown in this table. The principle component analysis eigenvalues and coefficients are shown in Table 3. A scatterplot of the specimens along the first and second axes (roots) of the principle components analysis results is shown in Figure 3. The Discriminant Function Analysis (DFA) assignment of each specimen is shown in Table 4.

3.1.1. Lethrinus erythropterus

The majority of the 21 characters were observed significantly ($P< 0.05$) for *L. erythropterus* with the exception of the character A3 (the posteriormost point of the eye to the origin of the dorsal fin); however, there was significant grouping of *L. erythropterus* populations by site (Table 2). The Manado and Wakatobi populations resembled each other for the characters A2, A6, B1, B3, B4, C1, and C4. The Makassar *L. erythropterus* population

differed from the other two locations for morphometric characters A4, A5, B5, B6, C3, C5, C6, D1, D3, D4, D5, and D6.

Standardized coefficients of canonical variables (roots) 1 and 2 from the DFA showed 95.4% and 41.7% variation between the populations analysed, with 20 principal variables for discriminating between groups (Table 3). In canonical root 1, the variables with major effects were: A6 (Y=1.304), B1 (Y=0.659), B3 (Y=0.323), B5 (Y=0.674), C3 (Y=0.782), C5 (Y=-0.362), C6 (Y=-0.365), D4 (Y=-0.373), (Y=0.713), D6 (Y=0.713). For root 2, variables with major effects were: A2 (Y=-0.388), A3 (Y=-0.423), A4 (Y=-0.564), A5 (Y=1.328), B5 (Y=-0.347), C3 (Y=0.354), C4 (Y=-0.473), C5 (Y=0.391), D1 (Y=0.504), D3 (Y=0.495), D4 (Y=-538).

Predictive classification assigned 93.3% of the 90 *L. erythropterus* specimens to their population of origin (Table 4). The *L. erythropterus* collected from Manado contained 3 specimens assigned to the Wakatobi group and vice-versa. Meanwhile, the *L. erythropterus* from Makassar formed a distinct group (100% assignment to place of origin) .

3.1.2. Lethrinus semicintus

The one-way ANOVA showed that certain characters contributed significantly ($p<0.05$) to between population differences in *L. semicintus* (Table 2). The Tukey post-hoc test showed significant differences between populations, except for the character C1, the origin of the anal fin to the insertion of the anal fin ($P > 0.05$). The Manado and Wakatobi populations were similar for the characters A1, A2, A5, B4, B5, C4, C5, C6, D1, D3, D4, D5, and D6. The Makassar population was significantly different from the other two populations for characters A3, A4, A6, B1, B3, B6, while character C3 was similar for the Makassar and Wakatobi populations (Table 2).

The standardized coefficients of the canonical variable (root) 1 and 2 obtained from the DFA show 96.04% and 50.41% variation between the populations analysed, with 19 principal variables for discriminating between groups (Table 3). The morphometric characters influencing root 1 where: A4 (Y=-0.316), A6 (Y=1.078), B1 (Y=0.744), B5 (Y=0.587). The variables with the strongest influence on root 2 were: A2 the posteriormost point of the maxilla to the posteriormost point of the eye (Y=-0.840), A3 (Y=-0.448), A4 (Y=0,541), A6 (Y=0.322), B1 (Y=0.336), B5 (Y=0.683), B6 (Y=-0.895), C1 (Y=0.482), C4 (Y=-0.317), D1 (Y=0.333), D3 (Y=868), D4 (Y=0.542), D5 (Y=-0.531), D6 (Y=-0.565).

Predictive classification of individuals showed an overall 97.8% assignment of *L. semicintus* to population of origin (Table 4). The DFA assigned one individual from the Manado population of *L. semicintus* to the Wakatobi population and vice-versa, while the *L. semicintus* population from Makassar clustered apart from the other two populations with 100% assignment to site of origin.

3.1.3. Lethrinus obsoletus

The ANOVA showed a significant ($p<0.05$) effect of population (site) on morphometric characters; the Tukey post-hoc test determined the level of significance for each parameter (Table 2). There was a significant difference ($P < 0.05$) between the three *L. obsoletus* populations for eight characters (A4, A5, A6, B1, B5, C6, D4, and D5). However, the difference was not significant between the Manado and Wakatobi populations for characters A2, B3, B6, C1, C3, C5, D1, D3, D6, and between the Manado and Makassar populations for characters A1, A3, and C4.

The standardized coefficients of canonical variables (roots) 1 and 2 from the DFA for *L. obsoletus* showed 90.25% and 49% variation between the populations analysed, with 22 principal discriminant group variables. The variables with the greatest influence on Root 1 were: A1 (Y=0.587), A3 (Y=0.310), A4 (Y=0.318), A6 (Y=0.520), B1 (Y=0.544), B5 (Y=0.645), C1 (Y=0.621), C4 (Y=-0.449), D1 (Y=0.379), D3 (Y=0.672), D6 (Y=0.347). The variables with the greatest influence on Root 2 were: A2 (Y=0.587), A3 (Y=0.516), A4 (Y=0.988), A5 (Y=-1.072), B5 (Y=0.687), C3 (Y=-0.562), C4 (Y=0.625), C5 (Y=-0.509), D4 (Y=0.572), D5 (Y=-0.443).

Predictive classification assigned 94.4% of the 90 *L. obsoletus* sampled to their population of origin (Table 4). Two individuals from the Manado *L. obsoletus* population were assigned to the Wakatobi population, and vice-versa. One individual from the Makassar population was assigned to the Manado group.

3.1.4. Lethrinus ornatus

The one-way ANOVA showed a significant ($P < 0.05$) between-site difference for the 21 morphometric characters. The Tukey post-hoc test did not show significant variation between the Manado and Wakatobi populations of *L. ornatus* for characters A1, A2, A3, B1, C1, C3, C5, D1, D3, D5, D6, while for character D4 the difference between Makassar and Wakatobi populations was not significant. However, nine characters differed significantly between all three populations (A4, A5, A6, B3, B4, B5, B6, C4, and C6).

The standardized coefficient of DFA canonical variables (roots) 1 and 2 showed 90.25% and 32.49% variation between the three *L. ornatus* populations, with 14 principal discriminant variables. The morphometric characters influencing Root 1 were: A6 (Y=0.934), B1 (Y=0.443), B4 (Y=-0.517), B6 (Y=0.353), C4 (Y=-0.370), C6 (Y=0.568), D1 (0.526). The characters with the greatest influence on Root 2 were: A1 (Y=0.342), B1 (Y=0.340), B6 (Y=-0.560), C1 (Y=0.408), C3 (Y=0.332), C6 (-0.370), and D3 (Y=0.409).

Predictive classification assigned 95.6% of the 90 *L. ornatus* individuals to their site of origin (Table 4). The Manado population of *L. ornatus* (93.3%) had 2 individuals assigned to the Wakatobi group and vice-versa. The Makassar population formed a distinct group with 100% assignment to site of origin.

3.1.5. Lethrinus harak

The one-way ANOVA of the 21 morphometric characters *L. harak* showed significant ($P< 0.05$) differences between the three populations at the 95% confidence level, indicating a significant effect of population on shape for this species. The Tukey post-hoc test (Table 2) showed that the Manado and Wakatobi population had similar values for seven characters (A1, A3, B1, B3, C4, D1, and D4), while Makassar and Wakatobi populations had similar values for three characters (C1, C3, and C5); however, character D3 differed significantly between the Makassar and Wakatobi populations. The values of ten characters (A2, A4, A5, A6,

B4, B5, B6, C6, D5, and D6) differed significantly between all three species.

The standardized coefficients of canonical components (roots) 1 and 2 from the DFA explained 90.25% and 32.49% of between population variation, with 14 principal discriminant group variables (Table 3). The morphometric characters influencing Root 1 values were: A6 (Y=0.934), B1 (Y=0.443), B4 (Y=-0.517), B6 (Y=0.353), C4 (Y=-0.370), C6 (Y=0.568), and D1 (Y=0.526). The main characters influencing Root 2 values were: A1 (Y=0.342), B1 (Y=0.340), B3 (Y=-0.323), B6 (Y=-0.560), C1 (Y=0.408), C3 (Y=0.332), C6 (Y=-0.370), and D3 (Y=0.409).

Predictive classification assigned 91.1% of the 90 *L. harak* individuals to their site of origin (Table 4). From the Manado population, one individual was assigned to the Wakatobi population. From the Wakatobi population, four individuals were assigned to Manado and one to Makassar. Two individuals from Makassar were assigned to the Wakatobi group.

Table 2. Variation (mean and standard deviation) in the morphometric characters of Lethrinus spp. from Sulawesi, Indonesia

Code	*L. erythropterus*			*L. semicintus*			*L. obsoletus*			*L. ornatus*			*L. harak*		
	Manado	Makassar	Wakatobi	Manado	Makassar	Wakatobi	Manado	Makassar	Wakatobi	Manado	Makassar	Wakatobi	Manado	Makassar	Wakatobi
A1	46.33 ± 2.69^{a}	51.69 ± 2.10^{b}	48.30 ± 4.36^{c}	47.37 ± 7.60^{a}	50.27 ± 1.69^{b}	45.89 ± 2.39^{a}	47.55 ± 1.45^{a}	48.56 ± 2.05^{a}	46.24 ± 2.35^{b}	41.84 ± 1.46^{a}	45.05 ± 2.26^{b}	41.55 ± 2.01^{a}	44.68 ± 3.57^{a}	48.81 ± 2.21^{b}	45.89 ± 2.58^{a}
A2	57.64 ± 3.47^{a}	64.60 ± 2.74^{b}	58.06 ± 3.04^{a}	49.61 ± 2.75^{a}	55.37 ± 1.21^{b}	49.03 ± 2.19^{a}	50.32 ± 3.49^{a}	52.94 ± 1.50^{b}	49.39 ± 1.44^{a}	50.40 ± 2.80^{a}	56.12 ± 2.17^{b}	49.21 ± 2.11^{a}	47.22 ± 3.86^{a}	53.40 ± 1.76^{b}	49.82 ± 2.20^{c}
A3	26.17 ± 8.76^{a}	28.44 ± 1.98^{a}	25.49 ± 1.91^{a}	22.49 ± 3.35^{a}	25.31 ± 1.44^{b}	20.93 ± 1.75^{c}	26.78 ± 6.67^{a}	25.90 ± 1.53^{a}	23.17 ± 1.32^{b}	23.41 ± 1.62^{a}	25.47 ± 2.12^{b}	22.40 ± 2.46^{a}	26.17 ± 4.21^{a}	30.10 ± 1.91^{b}	26.98 ± 2.77^{a}
A4	72.72 ± 2.03^{a}	83.75 ± 3.44^{b}	75.40 ± 3.70^{c}	57.96 ± 1.71^{a}	64.07 ± 2.40^{b}	56.55 ± 2.23^{c}	63.92 ± 1.29^{a}	65.59 ± 1.88^{b}	61.93 ± 2.27^{c}	65.96 ± 1.87^{a}	70.60 ± 2.92^{b}	62.89 ± 1.91^{c}	61.36 ± 5.05^{a}	69.88 ± 3.24^{b}	64.69 ± 3.69^{c}
A5	69.15 ± 1.93^{a}	77.97 ± 2.30^{b}	71.80 ± 3.64^{c}	56.86 ± 1.85^{a}	62.70 ± 2.11^{b}	55.78 ± 1.68^{a}	61.34 ± 1.44^{a}	62.91 ± 1.33^{b}	60.25 ± 1.85^{c}	63.12 ± 1.73^{a}	67.60 ± 2.16^{b}	60.92 ± 1.83^{c}	59.51 ± 5.32^{a}	67.21 ± 2.47^{b}	62.61 ± 3.33^{c}
A6	74.49 ± 4.93^{a}	86.77 ± 2.39^{b}	75.89 ± 3.94^{a}	65.39 ± 2.30^{a}	74.34 ± 1.58^{b}	64.21 ± 1.69^{c}	69.52 ± 2.55^{a}	73.50 ± 2.13^{b}	66.86 ± 1.72^{c}	67.11 ± 1.86^{a}	74.36 ± 2.45^{b}	64.61 ± 1.65^{c}	66.46 ± 5.48^{a}	75.09 ± 2.45^{b}	69.15 ± 3.84^{c}
B1	53.64 ± 3.45^{a}	58.90 ± 4.35^{b}	53.84 ± 2.93^{a}	48.93 ± 3.51^{a}	52.08 ± 2.77^{b}	46.92 ± 2.21^{c}	50.74 ± 2.21^{a}	54.44 ± 2.89^{b}	49.13 ± 2.17^{c}	46.57 ± 2.57^{a}	48.64 ± 3.11^{b}	45.57 ± 2.45^{a}	49.49 ± 3.98^{a}	56.63 ± 2.91^{b}	51.38 ± 2.53^{a}
B3	58.67 ± 2.37^{a}	65.86 ± 2.35^{b}	59.34 ± 3.21^{a}	52.63 ± 1.93^{a}	58.61 ± 3.63^{b}	49.72 ± 2.66^{c}	54.08 ± 1.41^{a}	56.07 ± 1.23^{b}	53.21 ± 2.25^{a}	52.97 ± 1.65^{a}	55.73 ± 2.00^{b}	50.87 ± 1.76^{c}	52.60 ± 3.73^{a}	57.44 ± 1.73^{b}	53.79 ± 3.44^{a}
B4	67.24 ± 1.79^{a}	76.56 ± 2.79^{b}	69.47 ± 5.40^{a}	50.78 ± 1.29^{a}	55.92 ± 1.25^{b}	50.32 ± 1.79^{a}	58.76 ± 1.22^{a}	59.80 ± 1.80^{ab}	56.42 ± 7.37^{ac}	60.73 ± 1.54^{a}	64.63 ± 2.74^{b}	59.24 ± 2.00^{c}	56.94 ± 4.44^{a}	64.55 ± 1.90^{b}	61.33 ± 3.84^{c}
B5	88.50 ± 1.92^{a}	101.06 ± 3.13^{b}	90.62 ± 3.51^{c}	73.14 ± 4.68^{a}	81.00 ± 1.50^{b}	72.43 ± 1.68^{a}	80.92 ± 1.63^{a}	83.11 ± 1.41^{b}	79.16 ± 1.91^{c}	81.07 ± 1.17^{a}	85.34 ± 3.98^{b}	78.94 ± 1.67^{c}	78.38 ± 5.54^{a}	86.84 ± 2.19^{b}	82.22 ± 4.24^{c}
B6	89.76 ± 1.98^{a}	101.10 ± 4.07^{b}	91.97 ± 3.44^{c}	74.08 ± 1.86^{a}	80.87 ± 2.40^{b}	70.93 ± 2.18^{c}	79.77 ± 1.78^{a}	82.97 ± 2.13^{b}	78.66 ± 3.30^{a}	80.11 ± 3.01^{a}	84.33 ± 2.89^{b}	76.18 ± 1.69^{c}	77.61 ± 5.98^{a}	88.52 ± 2.80^{b}	81.22 ± 4.54^{c}
C1	34.80 ± 3.09^{a}	39.05 ± 3.06^{b}	36.23 ± 1.74^{a}	31.41 ± 9.57^{a}	33.38 ± 1.01^{a}	30.15 ± 1.01^{a}	32.64 ± 0.77^{a}	33.85 ± 0.86^{b}	32.35 ± 0.89^{a}	32.48 ± 1.23^{a}	34.93 ± 1.21^{b}	32.86 ± 5.24^{a}	32.96 ± 2.98^{a}	36.54 ± 1.37^{b}	35.85 ± 2.07^{b}
C3	31.95 ± 0.96^{a}	36.61 ± 2.46^{b}	34.83 ± 3.25^{c}	29.85 ± 2.38^{a}	32.47 ± 1.49^{b}	32.41 ± 3.27^{b}	31.98 ± 0.85^{a}	33.08 ± 0.79^{b}	32.46 ± 1.31^{a}	32.99 ± 1.01^{a}	34.58 ± 2.15^{b}	33.08 ± 1.94^{a}	32.58 ± 2.80^{a}	37.01 ± 0.92^{b}	37.45 ± 3.33^{b}
C4	32.97 ± 1.47^{a}	37.24 ± 1.54^{b}	33.36 ± 1.59^{a}	28.14 ± 4.01^{a}	30.21 ± 3.32^{b}	26.35 ± 0.92^{a}	30.64 ± 0.86^{a}	30.96 ± 1.01^{a}	29.91 ± 1.09^{b}	29.33 ± 0.91^{a}	30.77 ± 1.20^{b}	28.51 ± 1.04^{c}	29.83 ± 2.86^{a}	32.79 ± 1.97^{b}	31.04 ± 1.95^{a}
C5	57.73 ± 1.63^{a}	65.36 ± 2.48^{b}	60.48 ± 3.21^{c}	48.20 ± 1.01^{a}	52.69 ± 1.12^{b}	49.16 ± 2.42^{a}	54.34 ± 1.02^{a}	55.36 ± 1.32^{b}	54.10 ± 1.57^{a}	54.92 ± 1.36^{a}	57.74 ± 2.30^{b}	53.99 ± 2.09^{a}	53.26 ± 4.12^{a}	60.63 ± 1.30^{b}	58.97 ± 3.83^{b}
C6	58.03 ± 1.29^{a}	65.56 ± 2.56^{b}	61.14 ± 8.25^{c}	47.14 ± 0.90^{a}	52.35 ± 1.38^{b}	46.58 ± 1.41^{a}	52.63 ± 1.14^{a}	54.37 ± 1.72^{b}	51.76 ± 1.18^{c}	52.46 ± 1.11^{a}	55.70 ± 1.70^{b}	50.72 ± 1.46^{c}	51.60 ± 4.70^{a}	57.90 ± 1.15^{b}	55.17 ± 4.01^{c}
D1	26.42 ± 2.26^{a}	32.70 ± 2.61^{b}	29.00 ± 1.89^{c}	28.70 ± 1.77^{a}	31.87 ± 1.99^{b}	28.64 ± 2.44^{a}	28.39 ± 1.59^{a}	31.32 ± 1.69^{b}	28.41 ± 1.49^{a}	26.40 ± 1.59^{a}	28.78 ± 1.74^{b}	25.95 ± 1.16^{a}	29.91 ± 3.28^{a}	33.12 ± 2.28^{b}	30.86 ± 2.36^{a}
D3	26.36 ± 2.04^{a}	30.04 ± 1.81^{b}	28.75 ± 2.37^{c}	27.07 ± 1.89^{a}	29.31 ± 1.70^{b}	28.10 ± 2.19^{a}	27.30 ± 1.74^{a}	30.48 ± 1.48^{b}	27.99 ± 1.42^{a}	25.99 ± 2.12^{a}	28.55 ± 2.42^{b}	26.58 ± 1.73^{a}	29.07 ± 2.84^{a}	31.13 ± 2.07^{b}	30.13 ± 2.17^{ab}
D4	25.19 ± 1.35^{a}	29.03 ± 1.33^{b}	26.17 ± 1.21^{c}	19.61 ± 0.83^{a}	22.69 ± 1.20^{b}	19.95 ± 0.99^{a}	23.45 ± 0.82^{a}	24.61 ± 0.71^{b}	22.75 ± 0.77^{c}	22.64 ± 5.68^{a}	23.38 ± 1.01^{ab}	21.08 ± 0.87^{ac}	22.46 ± 2.21^{a}	25.51 ± 1.21^{b}	23.27 ± 2.12^{a}
D5	38.26 ± 1.55^{a}	44.33 ± 1.92^{b}	40.24 ± 2.50^{c}	35.43 ± 1.87^{a}	38.99 ± 1.04^{b}	34.50 ± 1.75^{a}	37.73 ± 1.01^{a}	40.74 ± 1.42^{b}	37.82 ± 1.45^{c}	35.55 ± 1.67^{a}	38.84 ± 1.45^{b}	35.30 ± 1.83^{a}	37.67 ± 3.49^{a}	42.27 ± 2.01^{b}	39.66 ± 2.83^{c}
D6	39.91 ± 1.57^{a}	46.57 ± 2.25^{b}	41.73 ± 1.49^{c}	37.15 ± 1.45^{a}	40.64 ± 1.41^{b}	36.54 ± 1.43^{a}	39.11 ± 1.76^{a}	42.12 ± 1.58^{b}	38.28 ± 1.30^{a}	35.97 ± 2.13^{a}	39.09 ± 1.88^{b}	35.42 ± 1.01^{a}	39.71 ± 3.42^{a}	43.67 ± 2.12^{b}	$40.23 \quad 1.99^{a}$

*One-way ANOVA with Tukey post-hoc test; different superscript letters indicate significant difference at α= 0.05

Table 2. Eigenvalue, cumulative variance (%), canonical correlation, and standardized coefficients of the canonical variables produced by the principle components analysis of morphometric characters for Lethrinus species from Sulawesi, Indonesia

Species	*L. erythropterus*		*L. semicintus*		*L. obsoletus*		*L. ornatus*		*L. harak*	
Discriminant Variables	Function		Function		Function		Function		Function	
	1	2	1	2	1	2	1	2	1	2
Eigenvalue	21.127	0.71	24.46	1.02	10.47	0.98	11.20	0.49	11.2	0.49
Cumulative% of variance	96.7	3.3	96	4	91.4	8.6	95.8	4.2	95.8	4.2
Canonical correlation	0.977	0.646	0.98	0.71	0.95	0.70	0.95	0.57	0.95	0.57
A1	.001	-.130	.249	-.083	.587	.070	.185	.342	.185	.342
A2	.264	-.388	-.241	-.840	.140	.587	.263	.198	.263	.198
A3	.678	-.423	-.071	-.448	.310	.516	.130	-.272	.130	-.272
A4	-.183	-.564	-.316	.541	.318	.988	-.273	-.106	-.273	-.106
A5	.199	1.32	-.251	-.012	-.248	-1.072	.062	.007	.062	.007
A6	1.304	-.024	1.078	.322	.520	.014	.934	.006	.934	.006
B1	.659	.149	.744	.336	.544	-.053	.443	.340	.443	.340
B3	.323	-.280	.128	-.010	-.244	-.270	.212	-.323	.212	-.323
B4	-.268	.092	-.024	-.085	-.203	-.111	-.517	.291	-.517	.291
B5	.674	-.347	.587	.683	.645	.687	.004	.100	.004	.100
B6	-.188	.090	.069	-.895	.058	-.294	.353	-.560	.353	-.560
C1	.189	.087	.717	.482	.621	-.267	-.049	.408	-.049	.408
C3	.782	.354	.165	.154	.250	-.562	.002	.332	.002	.332
C4	-.227	-.473	-.021	-.317	-.449	.625	-.370	-.041	-.370	-.041
C5	-.362	.391	-.060	.254	-.453	-.509	-.154	-.227	-.154	-.227
C6	-.365	.021	.508	.206	-.151	-.094	.568	-.370	.568	-.370
D1	.177	.504	.153	.333	.379	-.043	.526	.241	.526	.241
D3	-.216	.495	.157	.868	.672	-.021	.129	.409	.129	.409
D4	-.373	-.538	.156	.542	-.167	.572	.043	-.259	.043	-.259
D5	.270	.078	.289	-.531	-.002	-.443	.067	.197	.067	.197
D6	.713	-.109	-.002	-.565	.347	-.282	-.149	.246	-.149	.246

Table 4. Predictive classification of individuals for five *Lethrinus* species from Sulawesi

Species	Collection site	Number of specimens assigned			Assignment (%)		
		Manado	Makassar	Wakatobi	Manado	Makassar	Wakatobi
L. erythropterus	Manado	27	0	3	90.0	0.0	10.0
	Makassar	0	30	0	0.0	100.0	0.0
	Wakatobi	3	0	27	10.0	0.0	90.0
L. semicinctus	Manado	29	0	1	96.7	0.0	3.3
	Makassar	0	30	0	0.0	100.0	0.0
	Wakatobi	1	0	29	3.3	0.0	96.7
L. obsoletus	Manado	28	0	2	93.3	0.0	6.7
	Makassar	1	29	0	3.3	96.7	0.0
	Wakatobi	2	0	28	6.7	0.0	93.3
L. ornatus	Manado	28	0	2	93.3	0.0	6.7
	Makassar	0	30	0	0.0	100.0	0.0
	Wakatobi	2	0	28	6.7	0.0	93.3
L. harak	Manado	29	0	1	96.7	0.0	3.3
	Makassar	0	28	2	0.0	93.3	6.7
	Wakatobi	4	2	25	13.3	6.7	83.3

3.1.6. Graphical analyses of morphometric characters

The distribution of the specimens along PCA components (roots) 1 and 2 based on morphometric characters indicates a close relationship between the five *Lethrinus* species (Fig. 3), with considerable overlap between species. In general, *L. erythropterus* specimens mostly grouped in the upper right-hand quadrant, while *L. semicintus* predominantly grouped in the lower left-hand quadrant, *L. obsoletus* straddled the lower right and left quadrants, *L. ornatus* mostly grouped in the upper left-hand quadrant, and *L.* harak straddled the upper and lower right-hand quadrants.

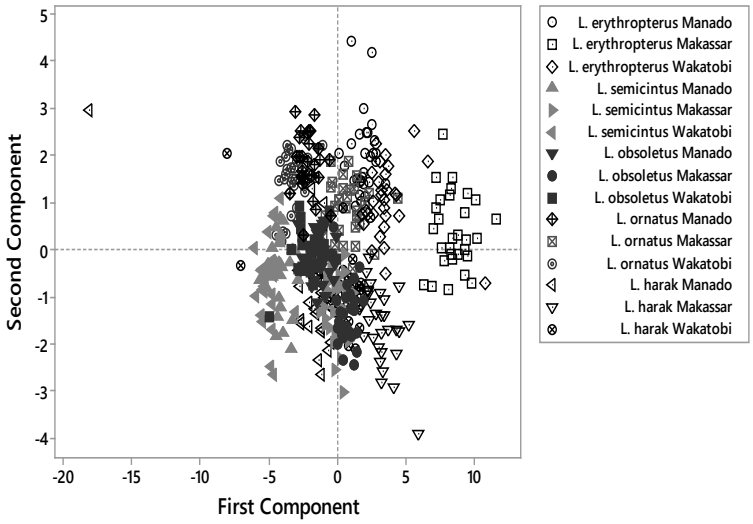

Figure 3. Scatterplot of the 420 specimens from three study locations and five species of the genus Lethrinus on the first component (Root 1) and second component (Root 2) of the principal component analysis based on 21 morphometric variables.

The tree analysis of the 21 morphometric variables (Fig. 4) shows distinct groupings based on the combination of species and study location. At the 66.67% level of similarity, four groups were formed: (1) L. erythropterus Manado, L. erythropterus Wakatobi, L. harak Makassar, L. ornatus Makassar; (2) L. erythropterus Makassar; (3) L. semicintus Manado, L. semicintus Wakatobi; (4) L. semicintus Makassar, L.obsoletus Makassar, L. obsoletus Manado, L. obsoletus Wakatobi, L. harak Manado, L. harak Wakatobi, L. ornatus Manado, L. ornatus Wakatobi. Despite this somewhat unexpected structure, for each of the five species the Manado and Wakatobi populations were relatively closely grouped with each other, but were not closely grouped with conspecifics from Makassar.

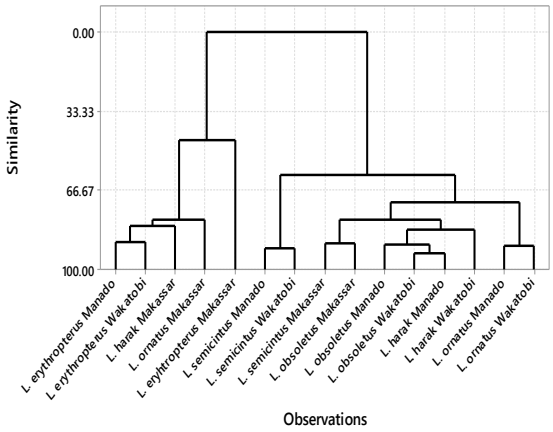

Figure 4. Dendrogram based on the similarity (Euclidean distance) of the 21 morphometric variables measured for five Lethrinus species from three study locations.

The canonical discriminant function analysis of group centroids showed a similar grouping pattern for the five species (Fig. 5). The Manado and Wakatobi specimens tended to be grouped to the left of the vertical axis, above or below the horizontal axis. Conversely, the Makassar populations were grouped to the right of the vertical axis. The centroid positions indicate that the first component predominantly discriminates between the Makassar population and the other two populations, while the second component predominantly discriminates between the Wakatobi and Manado populations.

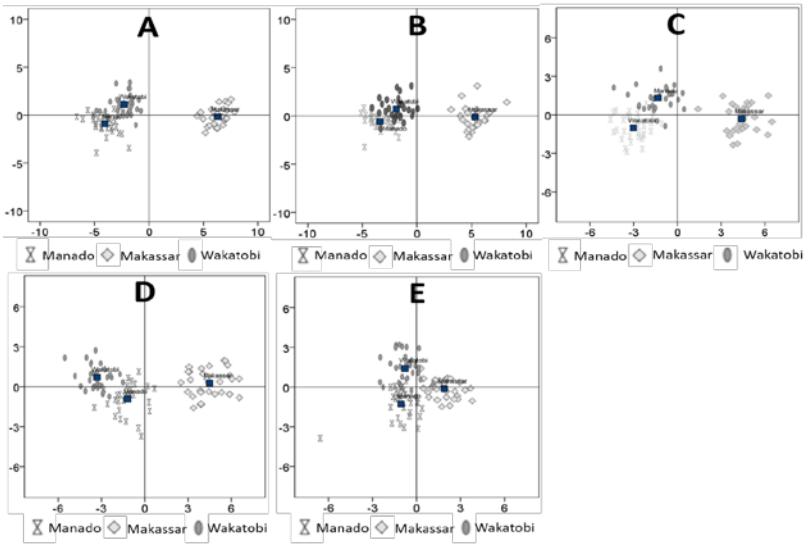

Figure 5. Distribution of the three Lethrinus populations studied based on discriminant analysis of morphometric characters for five species: **(A)** L. erythropterus: **(B)** L. semicintus: **(C)** L. obsoletus: **(D)** L. ornatus: **(E)** L. harak.

3.2. Meristic Character

The counts for the 8 meristic characters (Table 5) show that for four characters the five Lethrinus species had the same count range in all three study locations. These characters were: anal fin-rays, pectoral fin-rays, pelvic fin-rays, and number of gill-rakers. The dorsal spine count differed for L. obsoletus from Wakatobi with two values (IX, X), while for the remaining species and locations the dorsal spine count was always ten (X). The remaining three meristic counts (scales above lateral line, scales above lateral line, and scales below lateral line) varied between species and/or site.

Lateral line scale count varied more for *L. erythropterus* from Makassar (42-48) compared to the Manado and Wakatobi populations. Conversely, the scales above lateral line count varied more in the Manado population (4-6) compared to the other two populations. The number of scales below the lateral line was also more varied in Makassar (14-17) compared to the Manado and Wakatobi populations which both had the same range (14-16).

For *L. semicintus*, the Manado population had the widest range of lateral line scale count (46-50). The number of scales above the lateral line was most varied in the Wakatobi population (5-7), followed by Manado and Makassar. The scales below the lateral line count varied more in Manado (13-17) followed by Wakatobi, with once again the lowest variation in Makassar.

Table 5. Meristic characters of Lethrinus species from Sulawesi

Species	Locality	Dorsal fin-rays	Anal fin-rays	Pectoral fin-rays	Pelvic fin-rays	Lateral line scales	Scales above lateral line	Scales below lateral line	Number of gill-rakers
	Manado	X, 10	III, 9	12	I, 5	44-47	4, 6	14-16	4
L. erythropterus	Makassar	X, 10	III, 9	12	I, 5	42-48	4, 5	14-17	4
	Wakatobi	X, 10	III, 9	12	I, 5	44-47	5, 6	14-16	4
	Manado	X, 10	III, 9	12	I, 5	46-50	5, 6	13-17	4
L. semicintus	Makassar	X, 10	III, 9	12	I, 5	46-49	4, 5	13-14	4
	Wakatobi	X, 10	III, 9	12	I, 5	46-49	5, 7	13-16	4
	Manado	X, 10	III, 9	12	I, 5	45-48	5, 7	14-16	4
L. obsoletus	Makassar	X, 10	III, 9	12	I, 5	46-48	5	14-15	4
	Wakatobi	IX, X, 10	III, 9	12	I, 5	46-48	5, 6	14-16	4
	Manado	X, 10	III, 9	12	I, 5	45-48	5, 6	13-15	4
L. ornatus	Makassar	X, 10	III, 9	12	I, 5	45-47	5	14-15	4
	Wakatobi	X, 10	III, 9	12	I, 5	46-48	4, 6	13-16	4
	Manado	X, 10	III, 9	12	I, 5	43-49	5, 6	13-16	4
L. harak	Makassar	X, 10	III, 9	12	I, 5	43-48	5, 6	14-15	4
	Wakatobi	X, 10	III, 9	12	I, 5	45-48	5, 6	14-16	4

The meristic counts for *L. obsoletus* were most varied in Manado, and least varied in Makassar. In Manado, the lateral line scale count range was 45-48, while scales above lateral line count range was 5-7. For the latter character, the Makassar population was homogenous, with all specimens having 5 scales above the lateral line. The range of scales below lateral line count was the same for Manado and Wakatobi (14-16), wider than for Makassar (14-15).

For *L. ornatus*, the range of lateral line scales was greatest in Manado (45-48), and lowest in Wakatobi. Conversely, the Wakatobi population had the highest variation in scales above lateral line (4-6), and scales below lateral line (13-16), with the Makassar population having the lowest variation in both these counts.

For *L. harak*, scales above lateral line had the same range (5-6) at all three sites. Lateral line scale count and scales below lateral line count varied most in the Manado population (43-49 and 13-16), followed by Makassar for the former count and Wakatobi for the latter.

4. Discussion

The one-way analysis of variance (ANOVA) followed by Tukey post-hoc test and discriminant function analysis (DFA) showed that for most characters measured the difference between the Manado and Wakatobi populations was not significant ($P > 0.05$), while the Makassar population differed significantly for most characters ($P < 0.05$). The principal components analysis (PCA) and tree (dendrogram) analysis gave a similar result, showing close groupings of the Manado and Wakatobi populations, with the Makassar population distinctly separate.

The similarity in characters between Manado and Wakatobi, and difference with Makassar could be related to the differences in fishing pressure at each location. While there is a risk of destructive fishing in all waters around Sulawesi, the frequency is highest in Makassar, South Sulawesi Selatan, followed by Manado, North Sulawesi, and lowest in the Wakatobi, Southeast Sulawesi Tenggara (Briggs, 2003).

However, change in the dorsal fin-ray count of the species *L. obsoletus* was detected in the Wakatobi, the study location with the lowest level of destructive fishing. Differences in morphometric and meristic characters can occur in populations of fish which are of the same species but live in different locations (Firawati *et al*, 2017). Morphological changes in *Lethrinus* have been reported by Afrisal *et al* (2018) who found changes in the morphological characters of *L. erythropterus* in the Spermonde Archipelago, Makassar City, in particular spinal malformations.

Morphometric variation can be associated with or related to genetic variation (Hebert *et al*, 2003). Genetic analysis and research on parallel genetic structuring in the southwest Indian Ocean using the cytochrome c oxidase subunit I (COI) mtDNA gene and microsatellites (Healey *et al*, 2018a) found cryptic species within the genus *Lethrinus*, with different populations of some species (including *L. harak, L. mahsena* and *L. nebulosus*) appearing to be in fact different species. The species *L. harak* in this study is strongly divided based on morphometric traits, as shown in the tree analysis dendrogram (Figure 4). Similarly, genetic research revealed that *L. nebulosus* harvested in the Southwest Indian Ocean comprised in fact two cryptic species (Healey *et al*, 2018b). The emergence of cryptic species is thought to occur due to various reasons, including topography (Krakau 2008), anthropogenic activity and food availability (Irmawati *et al*, 2018).

The growth parameters have been calculated for *Lethrinus* species from different fishing grounds, including Southern Iran (Motlagh *et al*, 2010), the Gulf of Aden (Mehanna *et al*, 2014), the Persian Gulf (Raeisi *et al*, 2011), the Egyptian Red Sea (Zaahkouk *et al*, 2017), and within Indonesia in the Thousand Islands (Sevtian, 2012), Spermonde Archipelago (Budimawan *et al*, 2002), and Outer Kendari Bay (Nurdiansyah *et al*, 2017). For most of these populations, the growth pattern was allometric negative, with the exception of the Egyptian Red Sea, and Outer Kendari Bay populations which had isometric growth patterns. The availability of food is a crucial factor in determining growth patterns, and can lead to morphological changes in fish (Clabaut *et al*, 2007).

The results of this study indicate that destructive fishing may be a key driver of change in the morphometric characters of lethrinids in the waters around Sulawesi, and therefore that morphometric characters could serve as indicators of pressure from destructive fishing. Although destructive fishing activities may not affect individual lethrinids directly, there are several ways in which they could affect morphological characters. For example, the explosives used to catch fish can destroy around $0.5 – 2$ m^2 per 1 kg of "fish bomb" (McManus *et al*, 1997; Pet-Soede & Erdman, 1998). Destructive fishing can also cause mass mortality of fish and other marine organisms, which can lead to a reduction in biodiversity, local extinctions, and a reduction in predators, entraining changes in trophic structure as well as habitat modification (Bacalso and Wolff, 2014; Cinner, 2009). Degradation of coral reefs can promote increased algal growth, increasing food availability for herbivores while reducing the abundance of potential prey for carnivorous fishes (Piazzi *et al*, 2012). Such changes will affect the food and feeding habitats of the carnivorous lethrinids (Cinner, 2009). These factors can induce modifications in the physiology, reproductive patterns and feeding habit of fishes, which in turn can induce changes in fish body shape, such as bodily proportions and spine/ray counts (Alonzo *et al*, 2008; Unsworth *et al*, 2007), as observed in the five lethrinids in this study.

5. Conclusion

For all five species of the genus *Lethrinus* included in this study, the Manado and Wakatobi populations were similar, with little between-population variation in morphometric characters, while the Makassar populations differed substantially from the other two populations. The meristic scale counts (scales along lateral line, scales above lateral line, and scales below lateral line) varied most in the Manado populations and least in Wakatobi populations. Differences in destructive fishing levels could be associated with differences in morphometric characters. The *Lethrinus* populations within the Wakatobi National Park, exposed to the lowest level of destructive fishing, had less variation in meristic characters compared to the other two populations and some individuals of *L. obsoletus*

had one less spine in the dorsal fin compared to other *Lethrinus* populations and species.

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Bioactive Ingredients and Anti-influenza (H5N1), Anticancer, and Antioxidant Properties of *Urtica urens* L.

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Abstract

This study aims to emphasize the bioactive ingredients, antioxidant, anticancer, and anti-influenza (H5N1) effects of different extracts of *Urtica urens* L.. Three different extracts were prepared successively based on polarity yielding diethyl ether (DE), ethyl acetate (EA), and methanol (ME) extracts. GC-MS analysis revealed cyclopentasiloxane-decamethyl, quinine-1,1'-dioxide, and carotene as the prominent compounds in DE extract. The three extracts contain adequate amounts of total phenolics, total flavonoids, and total tannins and the superior amounts were detected in EA extract. Hesperidin, apigenin-6-arabinose-8-galactose, pyrogallol, and ferulic acid were the predominant individual phenolics identified and quantified in EA and ME extracts by HPLC, with a higher concentration in EA than in ME. The EA extract exhibited the highest antioxidant activity with IC_{50} 306.15±2.27 and 103.32±0.34 µg/ml for DPPH and ABTS assays, respectively and EC_{50} 352.50±2.50 µg/ml for reducing power assay. The DE and EA extracts have an efficient cytotoxicity effect against breast cancer cells (MCF-7) with IC_{50} values 28.9 and 48.58 µg/ml and colon cancer cells (HCT116) with IC_{50} values 39.81 and 41.21µg/ml, respectively. This is the first report regarding the antiviral effect of DE and ME extracts of *U. urens* against the avian influenza H5N1 virus. The DE exhibited the highest antiviral activity through the inhibition of H5N1 propagation with IC_{50} value 125 µg/ml. The results of the mode of action against H5N1 virus showed the potent virucidal effect of DE extract with 80% inhibition of viral propagation. The DE extract showed a limited effect on H5N1 viral replication and viral adsorption process. The DE extract of *Urtica urens* exhibited the highest anticancer and anti-influenza (H5N1) effects besides adequate antioxidant activity and is recommended for future use in the pharmaceutical applications for H5N1 and cancer (breast and colon) treatment.

Keywords: anticancer; antioxidant; avian influenza; H5N1; phenolic profile; *Urtica urens*.

1. Introduction

Currently, pharmaceutical industries depend on plant bioactive ingredients as a primer source of many drugs. Bioactive secondary metabolites of different medicinal plants including phenolic compounds, coumarins, flavonoids, alkaloids, terpenes, and tannins possess an array of beneficial biological properties to human health. Medicinal plants are one of the main sources of effective, safe, and economical therapeutics in several diseases (Mohamed *et al.,* 2016; Ali *et al.,* 2018).

Viral diseases infecting humans and animals cause serious health and economic issues. The prevention and the controlling of such viral diseases are so important to human health, and enormous research has been assigned to antiviral measures especially from natural products (Lee *et al.,* 2018). A variety of studies described the antiviral action of plant-derived polyphenols on herpes simplex virus (Moradi *et al.,* 2016), and rotavirus Wa, adenovirus type 7, and coxsackievirus B4 (Mohamed *et al.,* 2015). Worldwide, infection with influenza A virus is one of the serious problems that threaten human health and several animal species. Global seasonal influenza epidemics are responsible for 291,000- 646,000 deaths annually, depending on the virulence and transmissibility of the circulating viral strain (Sah *et al.,* 2019). Influenza A virus cannot be controlled due to its high level of genes mutation. Consequently, the avian influenza virus can be invasive for the human population at any time causing a considerable pandemic (Yang *et al.,* 2013). The high ability of the highly pathogenic avian influenza H5N1virus to infect and kill humans, besides the growing resistance to the currently used anti-influenza drugs such as oseltamivir and zanamivir, made the need to find new, natural, safe, and effective antiviral drugs against influenza virus crucial (Lee *et al.,* 2018). The antiviral effects of a number of compounds (gossypol, procyanidin B2-di-gallate, and Poly-galloylglucose) isolated from different medicinal plants against influenza viruses were reported (Lee *et al.,* 2018; Yang *et al.,* 2013; Derksen *et al.,* 2014; Ge *et al.,* 2014).

Cancer is a set of diseases provoked through uncontrolled cell life cycle producing abnormal and

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uncontrolled cell growth. It is one of the leading diseases that threaten human life worldwide, causing high levels of mortality more than coronary heart diseases or all strokes. Chemotherapy, hormone therapy, radiotherapy, and surgery are the present treatments of cancer. However, every one of these traditional treatments has very bad side effects (Islam *et al.,* 2018). The high death prevalence and the severe side effects of anticancer drugs motivated the scientists to explore safe and more efficient anticancer agents with minor side effects from natural sources. Recently, a wide array of plant-derived compounds was discovered to treat cancer, including vincristine isolated from the leaves of *Catharanthus roseus*, paclitaxel, isolated from the bark of *Taxus brevifolia*, besides many other compounds derived from different medicinal plants (Velde *et al.,* 2017; Seca and Pinto, 2018).

Oxidative stress, resulting from the imbalance of oxidative molecules and antioxidant substances in the body, causes the degeneration of tissues and cellular macromolecules. It induces inflammation, cancer, early aging, diabetes, and cardiovascular and neurodegenerative diseases, besides other negative issues that affect human health (Elnakish *et al.,* 2013). The utilization of plant-derived compounds as safe and more effective antioxidant agents was recommended through many investigations owing to its ability to neutralize and scavenge the harmful reactive oxygen species thus minimize oxidative harm of body tissues and avoid several diseases (Ali *et al.,* 2018; Gaafar *et al.,* 2018).

Urtica urens L. (stinging nettle), a perennial herb of family Urticaceae, is a native wild flowering plant that grows widely in North Africa, Europe, Asia, and North America. It is famous by the stinging hairs that line its stem, leaves, and flowers, and provoke irritation to the skin (Fu *et al.,* 2006). The general ancient therapeutic usage of stinging nettle especially in ancient Egyptians is the remedy of rheumatism, lumbago, muscular paralysis, and arthritis (Upton, 2013). The recent studies reported that Urtica spp. has antiviral, antiulcer, anti-inflammatory, antioxidant, anticancer, antimicrobial, analgesic, and immunomodulatory activities (Akbay *et al.,* 2003; Manganelli *et al.,* 2005; Upton, 2013; D'Abrosca *et al.,* 2019). The different categories of essential therapeutic phytoconstituent existing in Urtica plant are terpenoids (Ganber and Spiteller, 1995), flavonol glycosides including kaempferol-3-o-glucoside, and -3-O-rutinoside; quercetin-3-O-glucoside, and -3-O-rutinoside, isorhamnetin-3-O-glucoside, -3-O-rutinoside, and -3-O-neohesperidoside (Akbay *et al.,* 2003), and phenolic acids such as chlorogenic and caffeoyl malic acid (Pinelli *et al.,* 2008).

Preceding *in vitro* studies reported the cytotoxic effect of hydro-alcohol crud extracts of Urtica spp. in different cancer cell lines, including breast (Fattahi *et al.,* 2013), non-small cell lung cancer (NSCLC) (D'Abrosca *et al.,* 2019), and prostate cancers (Durak *et al.,* 2004). In addition, the antiviral effect of aqueous extract of Urtica spp. against the feline immunodeficiency virus (FIV) infection was reported (Manganelli *et al.,* 2005). Hitherto, to the best of our information, no prior investigations have studied the therapeutic potential effects of different extracts of *U. urens* against avian influenza H5N1 virus, breast, and colon cancer cells, along with its antioxidant activity. Thus, this study aims to investigate the potential anti-influenza (H5N1), anticancer, and antioxidant activities of diethyl ether, ethyl acetate, and methanol extracts of *U. urens*. Besides, this study is also set to identify the main bioactive secondary metabolites of these extracts to explore their potential contribution to such biological activities.

2. Materials and Methods

2.1. Plant material

The aerial part of *U. urens* was collected from the agricultural cultivated fields from El Sharkia government during February-2018. DR. Sameh Hussein identified the plant sample and deposited it in the Herbarium of the National Research Centre under the registered number 18975. Plant material was air-dried, then pulverized using a cutting mill.

2.2. Preparation of extracts

The pulverized plant material (100 g) was soaked with diethyl ether (1000 ml, 3 times) in 2000 ml conical flask and kept on an orbital shaker (Stuart, England) at 160 rpm at room temperature for 24 h. Then, the extract was filtered through filter paper Whatman No.1 to get the diethyl ether filtrate. This step was repeated twice, and the pooled filtrates of diethyl ether were concentrated using a vacuum rotary evaporator (Heidolph Unimax 2010, Germany) at 40°C to dryness giving diethyl ether extract (DE). After diethyl ether extraction, plant residue was dried and re-extracted with ethyl acetate (1000 ml, 3 times) following the same steps to give ethyl acetate extract (EA). Then plant residue after ethyl acetate extraction was dried and re-extracted with methanol (1000 ml, 3 times) following the same steps to give methanol extract (ME). The dried crude extracts were re-dissolved in dimethyl sulfoxide (DMSO) for further analysis.

2.3. Phytochemical analysis

2.3.1. GC mass spectrometry (GC-MS) analysis

The chemical profile of DE extract of *U. urens* was carried out using Thermo Scientific Capillary Gas Chromatography (model Trace GCULTRA) directly coupled to ISQ Single Quadruple MS and equipped with TG-5MS nonpolar 5% phenyl methylpolysiloxane capillary column (30 m × 0.25 mm ID × 0.25 μm). The operating condition of GC oven temperature was maintained as initial temperature 40^{o}C for 3 min, programmed rate 5^{o}C/min up to final temperature 280^{o}C with isotherm for 5 min. For GC-MS detection, an electronionization system with ionization energy of 70 eV was used. Helium was used as a carrier gas at a constant flow rate of 1.0 ml/min. Ten microliters of DE extract (1 mg/ml) were injected automatically in the splitless mode. Detection was performed in the full scan mode from 40 to 500 m/z. The quantification of the components was based on the total number of fragments (total ion count) of the metabolites as detected by the mass spectrometer. The identification of the chemical components was carried out based on the retention time of each component (Rt) compared with those of Wiley9 and NIST08 mass spectral libraries.

2.3.2. Total phenolic content

The total phenolic (TP) content was determined in DE, EA, and ME extracts of *U. urens* by Folin Ciocalteu reagent assay (Singleton and Rossi, 1965). A suitable aliquot (1 ml) of each extract was added to a 25 ml volumetric flask, containing 9 ml of distilled water. One milliliter of Folin Ciocalteu phenol reagent was added to the mixture and shaken. After 5 min, 10 ml of 7 % Na_2CO_3 solution was added to the mixture. The solution was diluted to 25 ml with distilled water and mixed. After incubation for 90 min at room temperature, the absorbance was determined at 750 nm with a spectrophotometer (Unicum UV 300) against prepared reagent as blank. A total phenolic content in the sample was expressed as mg Gallic acid equivalents (GAE)/g dry weight. All samples were analyzed in triplicate.

2.3.3. Total flavonoid content

The aluminum chloride method was used for the determination of total flavonoid (TF) content in DE, EA, and ME extracts of *U. urens* (Zhishen *et al.,* 1999). One milliliter of each extract was added to a 10 ml volumetric flask containing 4 ml of distilled water. To the flask, 0.3 ml 5 % $NaNO_2$ was added and after 5 min 0.3 ml 10 %, $AlCl_3$ was added. At 6th min, 2 ml of 1M NaOH were added and the total volume was made up to 10 ml with distilled water. The solution was mixed well, and the absorbance was measured against prepared reagent blank at 510 nm by using spectrophotometer (Unicum UV 300). The total flavonoid in the sample was expressed as mg quercetin equivalents (QE)/ g dry weight. All samples were analyzed in triplicate.

2.3.4. Total tannin content

Total tannin (TT) of DE, EA, and ME extracts of *U. urens* was measured using the Folin-Ciocalteu reagent methods (Polshettiwar *et al.,* 2007). One milliliter of each extract was added to 7.5 ml distilled water then add 0.5 ml of Folin reagent and 1 ml of 35% sodium carbonate solution. The volume was made up for 10 ml with distilled water and absorbance was measured against prepared blank reagent (all the reagents with 1 ml methanol instead of the extract) at 775 nm by using a spectrophotometer (Unicom UV 300, England). Total tannins in samples were expressed as mg tannic acid equivalent (TAE)/g dry weight.

2.3.5. HPLC analysis of individual phenolic and flavonoid compounds

The dried crude EA and ME extracts (10 mg) were dissolved in 2 ml methanol HPLC spectral grade by vortex mixing for fifteen minutes. The extract was filtrated through a 0.2µm Millipore membrane filter. The phenolic and flavonoid compounds were identified by HPLC (Agilent Technologies 1100 series, Germany), equipped with a quaternary pump (G131A model). The separation was achieved on ODS reversed-phase column (C18, 25×0.46 cm i.d. 5 µm, Netherlands). The injection volume (35 µl) was carried out with an auto sampling injector. The column temperature was maintained at 35°C. Gradient phenolic compounds' separation was carried out with an aqueous formic acid solution 0.1 % (A) and methanol (B) as a mobile phase at a flow rate of 0.3 ml/min following the method of (Goupy *et al.,* 1999). In addition, the flavonoid compounds' separation was carried out with 50 mM H_3PO_4, pH 2.5 (solution A) and acetonitrile (solution B) as a mobile phase at a flow rate of 0.7 ml/min as described by Mattila *et al.* (2000). Elutes were monitored using a UV detector set at 280 nm for the phenolic acids, and at 330 nm for flavonoids. Chromatographic peaks were identified by comparing the retention times with the respective retention times of known standard reference material. Phenolic acids and flavonoid compounds concentration were calculated by comparing its peak areas with the peak areas of used standards (with known concentration) based on the data analysis of Hewlett Packard software. Phenolic acids and flavonoid compounds were expressed as µg/g, DW.

2.4. Antioxidant capacity

2.4.1. DPPH• radical scavenging assay

The $DPPH^{\bullet}$ (0.1 mM) in methyl alcohol was prepared and 0.5 ml of this solution was added to 1 ml of DE, EA, and ME extracts of *U. urens* at different concentrations (100, 200, 300, and 400 µg/ml). The mixture was shaken vigorously and was allowed to stand at room temperature in the dark for thirty minutes. Butylhydroxytoluene (BHT, Sigma Aldrich, St. Louis, MO, USA) was used as a positive control, whereas the negative control contained the entire reaction reagent minus the extract. Then, the absorbance was measured at 515 nm against DMSO (Chu *et al.,* 2000). The capacity to scavenge the $DPPH^{\bullet}$ radical was calculated using the following equation:

$$DPPH^{\bullet} \text{ scavenging effect (\%)} = (A_c - A_s / A_c) \times 100$$

Where A_c is the absorbance of the control reaction and A_s is the absorbance in the presence of the plant extracts. The results were expressed as IC_{50} (the concentration (µg/ml) of the plant extract that scavenges 50 % of $DPPH^{\bullet}$ radical).

2.4.2. ABTS•+ antioxidant assay

Briefly, $ABTS^{\bullet+}$ was dissolved in double distilled water to 7.4 mM concentration, and potassium persulphate was added to a concentration of 2.6 mM. The working solution was prepared by mixing the two stock solutions in equal quantities. They were allowed to react for 12-16 hours at room temperature in the dark. The solution was then diluted by mixing 1 ml of the $ABTS^{\bullet+}$ solution with 60 ml of methanol to obtainabsorbance of 1.1 ± 0.02 at 734 nm using the spectrophotometer (Arnao *et al.,* 2001). The DE, EA, and ME extracts of *U. urens* (150 µl) at different concentrations (50, 100, 150, and 200 µg/ml) were allowed to react with 2850 µl of the freshly prepared $ABTS^{\bullet+}$ solution for two hours in the dark at room temperature. Then the absorbance was recorded at 734 nm. Trolox was used as a positive control. $ABTS^{\bullet+}$ scavenging activity (%) was calculated using the equation:

$$ABTS^{\bullet+} \text{ scavenging (\%)} = (A_0 - A_1) / A_0) \times 100$$

Where A_0 is the $ABTS^{\bullet+}$ absorbance of the control (the entire reaction reagent minus the sample), and A_1 is the $ABTS^{\bullet+}$ absorbance in the presence of the sample. The results were expressed as IC_{50} (the concentration (µg/ml) of the plant extract that scavenges 50 % of $ABTS^{\bullet+}$ radical).

2.4.3. Reducing power assay

The reducing power was assayed as described by Kuda *et al.* (2005). One milliliter of DE, EA, and ME extracts of *U. urens* at different concentrations (50, 100, 150, and 200

µg/ml) was mixed with 2.5 ml of phosphate buffer (50 mM, pH 7.0) and 2.5 ml of 1 % potassium ferricyanide. The mixture was then incubated at 50°C for twenty minutes. After the addition of 2.5 ml of trichloroacetic acid (10 %) to the mixture, centrifugation at 3000 rpm for ten minutes was performed. Finally, 1.25 ml from the supernatant was mixed with 1.25 ml of distilled water and 0.25 ml $FeCl_3$ solution (0.1 %, w/v). The absorbance was measured spectrophotometrically at 700 nm. BHT was used as positive control. The results were expressed as EC_{50} (the concentration (µg/ml) of the plant extract that provided the reading of 0.5 absorbance at 700 nm).

2.5. Cytotoxic activity

2.5.1. Cell lines and culture conditions

Two human carcinoma cell lines obtained from the Karolinska Institute, Stockholm, Sweden viz human breast cancer cells (MCF-7) and human colon cancer cells (HCT116) were used in this assay. The MCF-7 and HCT116 cancer cells were maintained in DMEM medium (LonzaBiowahittkar, Belgium). Media were supplemented with 1% antibiotic-antimycotic mixture (10,000 Uml-1 potassium penicillin, 10,000 µg/ml streptomycin sulphate, 25 µg/ml amphotericin B and 1% L-glutamine).

2.5.2. MTT assay

Cell viability was investigated using MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide] assay as published previously by Mosmann (1983). Cell lines were incubated in an incubator with 5% CO_2 at 37 °C (Sheldon, TC2323, Cornelius, OR, USA). Cells were plated into 96-well microplates at a concentration of 10^4 cells/well and allowed to stand for 24 h. The medium was aspirated, and fresh medium (without serum) was added to the cells with different concentrations (1.25-100 µg/ml) of DE, EA, and ME extracts of *U. urens* dissolved in DMSO. After 48h incubation, the medium was aspirated and 40 µl MTT solution (2.5 µg/ml DMSO) was added to each well and incubated for further 4 h. The final concentration of DMSO was less than 0.2%. The formazan crystals formed were dissolved and the reaction was stopped by adding 200 µl of 10% sodiumdodecyl sulfate (SDS) to each well for overnight at 37°C. The amount of formazan produced was measured at 595 nm with a reference wavelength of 620 nm as a background using a microplate reader (Bio-Rad Laboratories, model3350, USA). For the untreated cells (negative control), medium was added instead of the tested extracts. A positive control Adrinamycin® (doxorubicin) was used as a known cytotoxic natural agent giving 100% inhibition. Data were expressed as growth inhibition (%) using the following formula:

$$\text{Growth inhibition (\%)} = 100 - (A_{sample}/A_{control}) \times 100.$$

where; A_{sample} is the absorbance of treated cells with extract, and $A_{control}$ is the absorbance of untreated cells. The results were expressed as IC_{50} (the concentration (µg/ml) of the plant extract that inhibits 50% of cancer cell growth).

2.6. Antiviral activity

2.6.1. MTT assay

To determine TC_{50} value for each extract, MTT assay was applied for Madin Darby Canine Kidney (MDCK) cells as previously described by Mossman (1983) with minor modification. Briefly, MDCK cells were cultured in 96 well-plates (100 µl/well at a density of 3×10^5 cells/ml) and incubated for 24 h at 37°C in 5% CO_2. After 24 h, cells were treated with serially diluted extracts ranged from 100 - 800 µg/ml: in triplicates. After further 24 h, the supernatant was discarded, and cell monolayers were washed with sterile phosphate buffer saline (PBS) 3 times and MTT solution (20 µl of 5 mg/ml stock solution) was added to each well and incubated at 37 °C for 4 h followed by medium aspiration. In each well, the formed formazan crystals were dissolved with 200 µl of acidified isopropanol (0.04 M HCl in absolute isopropanol = 0.073 ml HCl in 50 ml isopropanol). The absorbance of formazan solutions was measured at λ_{max} 540 nm with 620 nm as a reference wavelength using a multi-well plate reader. The percentage of cytotoxicity compared to the untreated cells was determined with the following equation.

$$\text{\% Cytotoxicity} = (A_c - A_s/A_c) \times 100$$

Where; A_c: Absorbance of cells without treatment, and A_s: Absorbance of cells with treatment

Data were expressed as EC_{50} that represents the concentration of extract causing 50% cytotoxicity against MDCK cells corresponding to the control (without treatment).

2.6.2. Plaque reduction assay

The antiviral activities of DE, EA, and ME extracts of *U. urens* were determined by plaque reduction assay (Schuhmacher *et al.*, 2003). Briefly, MDCK cells were seeded in 6 well-culture plates (10^5cells/ml) and incubated for 24 h at 37°C in 5% CO_2. Previously titrated A/chicken/Egypt/B13825A/2017 (H5N1) virus was diluted to optimal virus dilution, which gave countable plaques/well, and mixed with the safe concentration of each tested extracts of *U. urens*. The virus was incubated for an hour at 37°C before being added to cells. The medium was removed from the 6-well cell culture plates and virus-compound mixtures inoculated in duplicate. After 1 h contact time for virus adsorption, 3 ml of DMEM supplemented with 2% agarose, 1% antibiotic antimycotic mixture, and 4% bovine serum albumin (BSA, Sigma) were added to the cell monolayer. The plates were left to solidify and incubated at 37°C until the formation of viral plaques (3 days). Formalin (10%) was added to each well for 1 h and the over layer removed. Fixed cells were stained with 0.1% crystal violet in distilled water. The untreated virus was included in each plate as a control. Finally, plaques were counted and the percentage reduction in virus count was recorded as follows:

% inhibition= viral count (untreated) - viral count (treated)/viral count (untreated) x 100

Data were expressed as IC_{50} that represents the concentration of extract causing 50% inhibition on the propagation of H5N1 virus corresponding to the control (without extract).

The selective index per extract was determined from the ratio of EC_{50}/IC_{50}

2.6.3. Mode of antiviral activity

2.6.3.1. Viral replication

The assay was carried out in a 6 well plate where MDCK cells were cultivated (10^5 cell/ml) for 24 h at 37°C. The virus was diluted to give 10^1 to 10^2 PFU/ well and

applied directly to the cultured cells and incubated for 1 hour at 37°C; unabsorbed viral particles were removed by washing cells three successive times by supplements free-medium. The effective plant extract was applied at different concentrations, after 1hour of contact time; 3 ml of DMEM medium supplemented with 2% agarose was added to the cell monolayer. Plates were left to solidify and incubated at 37°C till appearance of viral plaques. Cell monolayers were fixed in 10% formalin solution for 2 h and stained with crystal violet (Schuhmacher *et al.,* 2003). Control wells were included where MDCK cells were incubated with the virus, and finally plaques were counted and percentage reduction in plaques formation in comparison to control wells was recorded as mentioned above.

2.6.3.2. Viral adsorption

MDCK cells were cultivated in a 6 well plate (10^5cell/ml) for 24 h at 37 °C. The effective plant extract was applied at different concentrations in 200 µl medium without supplements and co-incubated with the cells for 2 h at 4 °C. The unabsorbed extract was removed by washing cells three successive times with supplements free-medium then A/chicken/Egypt/B13825A/2017 (H5N1) virus diluted to give 10 to 100 PFU/ well was co-incubated with the pretreated cells for 1 h followed by adding 3 ml DMEM supplemented with 2% agarose (Schuhmacher *et al.,* 2003). Plates were left to solidify then incubated at 37 °C to allow formation of viral plaques, fixed and stained as above mentioned to calculate percentage reduction in plaques formation in comparison to control wells where untreated MDCK cells were directly infected with influenza virus (H5N1).

2.6.3.3. Virucidal

The assay was carried out in a 6 well plate where MDCK cells were cultivated (10^5 cell/ml) for 24 h at 37 °C. A volume of 200 µl serum-free DMEM containing 10 to 100 PFU forming was added to the concentration of the effective plant extract, after 1-hour incubation, the mixture was diluted using serum-free medium 3 times each 10-fold that still allows existence of viral particles to grow on MDCK cells but leaves nearly no extract and 100 µl of each dilution were added to the MDCK cell monolayer. After 1h contact time, DMEM over layer was added to cell monolayer (Schuhmacher *et al.,* 2003). Plates were left to solidify then incubated at 37 °C to allow the formation of viral plaques, fixed and stained as above mentioned to calculate percentage reduction in plaques formation in comparison to control wells where the cell was infected with virus that was not pretreated with the tested extract.

2.7. Statistical analysis

All tests were conducted in triplicate. Data are reported as means ± standard deviation (SD).

Analysis of variance and significant differences among means were tested by one-way ANOVA using the COSTAT computer package. The least significant difference (LSD) at P≤0.05 level was calculated.

3. Results and Discussion

Recently, the therapeutic use of plant bio-active ingredients in complementary and alternative medicine for diverse disease treatment is now increasing worldwide. These natural bioactive components may act as new effective drugs for different kinds of harmful and mortal diseases or serve as an initial synthesizer for developing and designing new drugs for such diseases (Tringali, 2011). Different extracts of *U. urens* investigated in this study showed different bioactive ingredients including volatile compounds, phenolic acids and flavonoid compounds with different biological activities including antioxidants, anticancer and antiviral activities.

3.1. The GC-MS analysis of DE extract

The GC-MS analysis of DE extract showed the presence of different volatile bioactive components of different chemical categories (Table 1). Oxygenated compounds are the most category identified in DE extract including cyclopentasiloxane-decamethyl (CAS) (4.58%), dodecamethyl-cyclohexasiloxane (4.37%), and cycloheptasiloxane-tetradecamethyl (3.54%). Nitrogen-containing compounds were identified also in DE extract with an adequate percent including quinine-1,1'-dioxide (4.73%), 19,20-Didehydroyohimbinone (2.47%), and papaveroline (1.86%). Besides, hydrocarbon compounds (carotene, 1.05%) were identified in DE extract by GC-MS (Table 1). These results are in consensus with the results of Dar *et al.* (2013) who identified several volatile bioactive components including octadecan-1-ol, 4,6-di-tert-butyl-m-cresol, 2,4-ditertbutylphenol, n-cetane, dodecane, and other compounds in the hexane extract of *U. dioica* collected from India using GC-MS analysis. 19,20-Didehydroyohimbinone, identified in this study, was isolated from different species of genus Aspidosperma and is one of the β-carboline alkaloids that possess antimicrobial and antitumor effects (Layne *et al.,* 2015). Correspondingly, papaveroline, identified in this study, is an alkaloid compound isolated hitherto from opium, and earlier studies have confirmed its phosphodiesterase inhibition effects (Castellani and Zagaria, 1978). β-carotene the major hydrocarbon compound identified in DE extract of *U. urens* in this study was formerly reported in different Urtica species with its antioxidant activity (Upton, 2013).

Table 1. GC-MS analysis of diethyl ether (DE) extract of *U. urens.*

No.	RT	Compound Name	Area %	Molecular Formula
1	9.09	Papaveroline	1.86	$C_{16}H_{13}NO_4$
2	9.14	19,20-Didehydroyohimbinone	2.47	$C_{21}H_{22}N_2O_3$
3	9.22	3-Hydroxyandrosta-5,7,9(11)-trien-17-one	1.99	$C_{19}H_{24}O_2$
4	9.71	Ethyl 3,7,12-trihydroxycholan-24-oate	0.95	$C_{26}H_{44}O_5$
5	13.67	Cyclopentasiloxane, decamethyl (CAS)	4.58	$C_{10}H_{30}O_5Si_5$
6	16.40	3,5-Androstadien-17-one oxime	0.95	$C_{19}H_{27}NO$
7	17.36	7,8-Epoxylanostan-11-ol,3-acetoxy	1.06	$C_{32}H_{54}O_4$
8	18.37	Dodecamethylcyclohexasiloxane	4.37	$C_{12}H_{36}O_6Si_6$
9	19.68	Pregn-4-ene-3,11,20-trione,6,17,21-tris-[(trimethylsilyl)oxy],3,20-bis (Omethyloxime), (6á)	1.95	$C_{32}H_{58}N_2O_6Si_3$
10	20.53	Quinine 1,1'-dioxide	4.73	$C_{20}H_{24}N_2O_4$
11	22.63	Cycloheptasiloxane, tetradecamethyl	3.54	$C_{14}H_{42}O_7Si_7$
12	25.67	Penta-2,4-dien-1-one,5-dimethylamino-1-[5-(4-dimethylamino) buta-1,3-dienyl-2-thienyl]-	1.05	$C_{17}H_{22}N_2OS$
13	26.22	5-[(2,4-Dinitrophenyl) hydrazono] pentan-2-ol	1.03	$C_{11}H_{14}N_4O_5$
14	26.46	1,1,3,3,5,5,7,7,9,9,11,11,13,13,15,15-Hexadecamethyl-octasiloxane	2.38	$C_{16}H_{50}O_7Si_8$
15	29.76	4-(4-Chlorophenyl)-2-(cyclopropyl)-6-[4-benzylpiperazinyl-1-yl] benzonitrile	1.51	$C_{27}H_{26}ClN_3$
16	33.23	Plectaniaxanthin	0.99	$C_{40}H_{56}O_2$
17	35.33	Quercetin 7,3',4'-trimethoxy	1.07	$C_{18}H_{16}O_7$
18	36.53	2,15-Heptadecadiene,9-(ethoxymethyl)-	1.17	$C_{20}H_{38}O$
19	37.73	Acouenoside A	0.98	$C_{30}H_{46}O_9$
20	41.20	Docosahexaenoic acid, 1,2,3-propanetriyl ester	0.96	$C_{69}H_{98}O_6$
21	42.05	5á-Cholestan-3-one, cyclic ethylene acetal	1.36	$C_{29}H_{50}O_2$
22	47.00	Phorbol 12,13,20-triacetate	1.59	$C_{26}H_{34}O_9$
23	48.07	Carotene	1.05	$C_{40}H_{56}$
24	48.53	Ethyl isoallocholate	1.66	$C_{26}H_{44}O_5$
25	49.76	(14á)3,19-Epoxyandrosta-5,7-diene,4,4-dimethyl-3-methoxy-17-methylthiomethoxy	1.26	$C_{24}H_{36}O_3S$

3.2. Total phenolic (TP), total flavonoid (TF), and total tannins (TT) contents

The successive extraction of *U. urens* using polar gradient solvents of diethyl ether, ethyl acetate, and methanol showed a significant effect on the contents of TP, TF, and TT (Figure 1). Consistent with Folin-Ciocalteu test results, the highest amounts of TP and TT were detected in EA (160. 63±1.38 and 92.75±1.13 mg/g) followed by ME (68.10±0.63 and 42.56±0.83 mg/g), then DE (25.71±0.95 and 16.59±0.55mg/g), respectively. Correspondingly, the results of Aluminum chloride assay showed that the elevated amount of TF was detected in EA (51.67±0.37 mg/g), compared to ME (20.37±0.55mg/g), and DE (9.63±0.62mg/g) as presented in Figure 1. The contents of TP, TF, and TT of EA extract in this study were superior to the TP, TF, and TT contents of organic solvent extracts of different Urtica species from other countries (Mzid *et al.,* 2017; Zekovic *et al.,* 2017). This high content of TP, TF, and TT in EA extract is probably a result of the successive extraction of *U. urens* using polar gradient solvents that increased the liberation of more chemical compounds within different solvent fractions. A number of former studies reported the positive effect of successive extraction by polar gradient solvents on the high content of different bioactive compounds in different medicinal plants (Mohamed *et al.,* 2016; Zlotek *et al.,* 2016; Zekovic *et al.,* 2017).

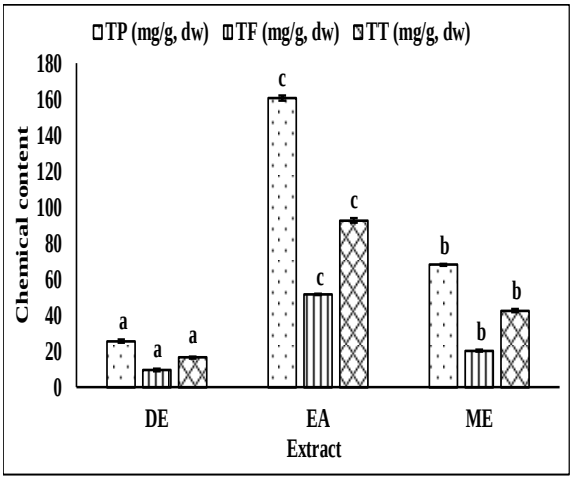

Figure 1. Content of total phenolic compounds (TP mg/g), total flavonoids (TF mg/g), and total tannins (TT mg/g) of diethyl ether (DE), ethyl acetate (EA), and methanol (ME) extracts of *U. urens.* Results are mean values ± standard deviations (n=3). Category bars with different letters are significantly different ($p \leq 0.05$).

3.3. HPLC identification and quantification of individual phenolics

Thirty-four individual phenolics were identified and quantified by HPLC in EA and ME extracts of *U. urens* (Table 2). Depending on the chemical structure of phenolic compounds, the identified phenolic compounds were corresponding to six groups namely benzoic acid

derivatives (6 compounds), cinnamic acid derivatives (7 compounds), flavonols (5 compounds), flavanone (4 compounds), flavones (7 compounds), and other phenols (5 compounds) as presented in Table 2. Excepting of gallic acid, benzoic acid, and luteolin-7-glucose, which were identified only in ME extract while all the other individual phenolics were identified in both EA and ME, with a higher concentration in EA than in ME (Table 2). This might be due to the more liberal of phenolic compounds after successive extraction and the more recovery effect of ethyl acetate on the phenolic individuals in *U. urens*. The positive effect of used solvent in the content of total phenolics and phenolic individuals was previously reported (Zlotek *et al.*, 2016; Zekovic *et al.*, 2017). As revealed in Table 2, benzoic acid derivatives (0.82 mg/g vanillic acid and 0.22 mg/g p-OH-benzoic acid), cinnamic acid derivatives (1.14 mg/g ferulic acid and 0.59 mg/g chlorogenic acid), flavonols (1.10 mg/g quercetin and 0.79 mg/g kaempferol), flavanone (6.54 mg/g hesperidin and 1.72 mg/g naringin), flavones (5.56 mg/g apigenin-6-arabinose-8-galactose and 1.11 mg/g acacetin-7-O-rutinoside), and other phenolics (1.25 mg/g pyrogallol and 0.97 mg/g caffeine) were the predominant individual phenolics identified in EA extract. The HPLC phenolic profile of *U. urens* in this study is in good accord with earlier results of Otles and Yalcin (2012) and Zekovic *et al.* (2017); they identified various phenolic compounds and flavonoids in *U. dioica* including gallic acid, ferulic acid, protocatechuic acid, kaempferol, quercetin, apigenin, naringenin, catechin, luteolin-7-O-glycoside, rutin, quercitrin, and other phenolics. Other findings identified and quantified varied contents of phenolic compounds and flavonoids in different extracts of Urtica spp. from different countries (Carvalho *et al.*, 2017; Mzid *et al.*, 2017; Vajic *et al.*, 2018). In this context, the contents of chlorogenic acid (15.3±0.21 mg/g) and rutin (6.01±0.09 mg/g) in *U. dioica* (Vajic *et al.*, 2018) were greater than their contents in *U. urens* in this study, while the contents of ferulic acid (1.14 mg/g), kaempferol (0.79 mg/g), quercetin (1.10 mg/g), apigenin (0.35 mg/g), and naringenin (0.62 mg/g) in *U. urens* were greater than their contents in *U. dioica* reported by Zekovic *et al.* (2017). This variation could be caused by varying geographical and ecological growing conditions where the plant has been collected (Hegazy *et al.*, 2019).

Table 2. HPLC identification and quantification of phenolic compounds of ethyl acetate (EA), and methanol (ME) extracts of *U. urens.*

Phenolic compound	Concentration (mg/g, extract)		Phenolic compound	Concentration (mg/g, extract)	
	E A	ME		EA	ME
Benzoic acid derivatives			flavanone		
Gallic acid	-	0.11	Narengin	1.72	0.20
Protocatchuic acid	0.21	0.18	Hesperidin	6.54	5.95
p- OH- benzoic acid	0.22	-	Naringenin	0.62	0.08
4-Amino-benzoic acid	0.12	0.04	Hespirtin	0.29	0.02
Benzoic acid	-	0.35	Flavones		
Vanillic acid	0.82	0.28	Apigenin-6-arabinose-8- galactose	5.56	5.60
Cinnamic acid derivatives			Apigenin-6-rhamnose 8- glucose	0.08	0.03
Chlorogenic acid	0.59	0.15	Luteolin-7-glucose	-	0.39
Caffeic acid	0.04	0.02	Apigenin-7-glucose	0.15	0.01
Rosmarinic acid	0.06	0.04	Apigenin-7-O-neohespiroside	0.85	0.20
Ferulic acid	1.14	0.10	Acacetin-7-O-rutinoside	1.11	0.39
Iso- Ferulic acid	0.14	0.04	Apigenin	0.35	0.02
α- Coumaric acid	0.05	0.03	Other simple phenols		
3,4,5-Methoxy-cinnamic acid	0.24	0.11	Pyrogallol	1.25	4.90
Flavonols			Caffeine	0.97	0.06
Quercetin	1.10	0.39	Coumarin	0.57	0.07
Kaempferol	0.79	0.24	Oleuropin	0.54	0.22
Catechein	0.69	0.25	Catechol	0.04	0.06
Quercetrin	0.49	0.05			
Rutin	0.31	0.05			

3.4. Antioxidant activity

The antioxidant activity of DE, EA and ME extracts of *U. urens* was assessed using DPPH, ABTS, and reducing power assays. The results are given in Table 3 and expressed as IC_{50} µg/ml for DPPH and ABTS and as EC_{50} µg/ml for reducing power. Consistent with DPPH, ABTS, and reducing power assays data, the EA extract exhibited the highest activity with IC_{50} 306.15±2.27 and 103.32±0.34 µg/ml for DPPH and ABTS assays, respectively and EC_{50} 352.50±2.50 µg/ml for reducing power assay followed by DE then ME; however, EA extract showed lower antioxidant activity than the antioxidant standards Trolox and BHT (Table 3). These results are in consensus with hitherto published findings stated the antioxidant activity of Urtica spp. using DPPH, ABTS and reducing power assays (Gulçin *et al.*, 2004; Otles and Yalcin, 2012; Carvalho *et al.*, 2017; Zekovic *et al.*, 2017). The high antioxidant activity of EA extract in this study might be owing to its high content of TP, TF, TT, and individual phenolics and flavonoids when compared to DE and ME extracts as presented in Figure 1 and Table 2. Numerous studies reported the association of TP, TF, TT, and individual phenolics and flavonoids with the antioxidant activities of different plants (Ibrahim *et al.*, 2015; Gaafar *et al.*, 2016; Ali *et al.*, 2018; Gaafar *et al.*, 2018). A significant variation among antioxidant activity of Urtica spp. extracts has been reported based on the used

solvent. Water extract of *U. dioica* exhibited reducing power activity (EC_{50} of 110 mg/ml) and DPPH scavenging activity (37%) at 60 µg/ml concertation (Gulçin *et al.,* 2004). The 50% aqueous ethanol extract of *U. dioica* exhibited DPPH scavenging activity (2.89±0.33 Trolox equivalents) and ABTS scavenging activity (2.60±0.14 Trolox equivalents) as descried by Carvalho *et al.* (2017). Besides, the 80% hydroalcoholic extract of *U. dioica* and its sub-fractions petroleum ether, ethyl acetate, n-butanol and water revealed DPPH scavenging activity with IC_{50} values 140, 215.96, 78.99, 168.24 and 302.90 µg/ml, respectively (Joshi *et al.*, 2015).

Table 3. Antioxidant activity: DPPH (IC_{50} µg/ml), ABTS (IC_{50} µg/ml), and reducing power (EC_{50} µg/ml) of diethyl ether (DE), ethyl acetate (EA), and methanol (ME) extracts of *U. urens.*

Extracts	IC_{50} µg/ml		EC_{50} µg/ml
	DPPH	ABTS	Reducing Power
DE	378.52^{c} ± 6.40	113.91^{c}± 1.26	382.67^{b}±0.58
EA	306.15^{b} ± 2.27	103.32^{b} ± 0.34	352.50^{c}±2.50
ME	696.21^{d} ± 2.18	220.68^{d} ± 0.12	678.33^{d}±1.44
BHT	3.30^{a} ± 0.18	-	3.29^{a} ± 0.14
Trolox	-	4.83^{a}±0.01	-

Results are mean values ± standard deviations (n=3). Means followed by the different letters in a column are significantly different ($p \leq 0.05$). Trolox and Butylated hydroxytoluene (BHT) was used as standard.

3.5. Anticancer activity

The MTT cytotoxicity assay data illustrated in Figure 2 indicated that DE and EA extracts have an efficient cytotoxicity effect against breast cancer cells (MCF-7) with IC_{50} values 28.9 and 48.58 µg/ml and colon cancer cells (HCT116) with IC_{50} values 39.81 and 41.21µg/ml, respectively as compared with ME extract. The present results are in accord with a number of previous studies indicated the cytotoxic effect of Urtica plant against different human cancer cells including breast (MCF-7) (Abu-Dahab and Afifi, 2007; Fattahi *et al.*, 2013), human colon cancer (HCT-116) and human prostate cancer (PC3) (Mohammadi *et al.*, 2016), human colon cancer (HT29) and human gastric cancer (MKN45) (Ghasemi *et al.*, 2016), prostate (Durak *et al.*, 2004), non-small cell lung cancer (NSCLC) cell lines (H460, H1299, A549 and H322) (D'Abrosca *et al.*, 2019), and human rhabdomyosarcoma (RD), human cervix carcinoma-HeLa derivative (Hep2c) and murine fibroblast (L2OB) (Zekovic *et al.*, 2017). Compared with the available results, the cytotoxic effect (IC_{50} values 28.9 and 48.58 µg/ml) of DE and EA extracts respectively, of *U. urens* in this study against breast cancer cells (MCF-7) is higher than that (IC_{50} value 2 mg/ml) of aqueous extract of *U. dioica* (Fattahi *et al.*, 2013), and higher than that of ethanol extract of *U. dioica* that caused about 6.88 ± 6.88 % growth inhibition of breast cancer cells (MCF-7) at 50 µg/ml concentration (Abu-Dahab and Afifi, 2007). Conversely, the cytotoxic effect (IC_{50} values 39.81 and 41.21 µg/ml) of DE and EA extracts respectively, of *U. urens* in this study against colon cancer cells (HCT116) is lower than that (IC_{50} value 23.61 µg/ml) of dichloromethane extract of *U. dioica* (Mohammadi *et al.*, 2016), also is lower than that (IC_{50} value 24.7 µg /ml) of ethanol extract of *U. dioica* roots against HT29 human colon cancer (Ghasemi *et al.*, 2016). The cytotoxic effect of the DE extract against MCF-7 and HCT116 cancer cells in this study could be owing to its richness of oxygenated and nitrogen-containing compounds (Table 1). Related to our findings D'Abrosca *et al.* (2019) attributed the antiproliferative effect of the *U. dioica* extract against small cell lung cancer (NSCLC) cell lines (H460, H1299, A549 and H322) to the presence of oxylipins as oxygenated compounds. Also, 19, 20-Didehydroyohimbinone, identified in DE extract (Table 1), is one of the β-carboline alkaloids that possess antimicrobial and antitumor effects (Layne *et al.*, 2015). Besides, the high cytotoxic effects of EA extract against MCF-7 and HCT116 cancer cells in these findings are parallel to its highest content of total phenolic, total flavonoids and total tannins (Figure 1) and its richness of individual phenolic acids and flavonoid compounds (Table 2), as well as to its highest antioxidant activities (Table 3). Thus, its cytotoxic properties could be owing to the presence of different phenolic acids and flavonoid compounds. This is in consensus with Konrad *et al.* (2000) who attributed the cytotoxic effect of *U. dioica* extract against prostate cancer cells to the presence of caffeic malic acid, caffeic acid, chlorogenic acid, and quercetin. A number of previous findings attributed the anticancer activities of different plant extracts to their high contents of total phenolic and total flavonoids besides different individuals of phenolics (Fattahi *et al.*, 2013; El Baz *et al.*, 2015; Gaafar *et al.*, 2018).

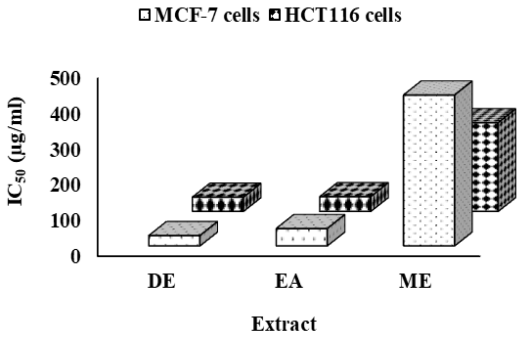

Figure 2. Cytotoxicity (IC_{50}, µg/ml) of diethyl ether (DE), ethyl acetate (EA), and methanol (ME) extracts of *U. urens* against breast cancer cells (MCF-7) and colon cancer cells (HCT116).

3.6. Antiviral activity

The cytotoxicity effect of DE, EA and ME extracts of *U. urens* against MDCK cells was detected by MTT assay. The data illustrated in Table 4 indicated that EA extract showed a cytotoxic effect against MDCK with TC_{50} value 54.49 µg/ml, while DE and ME were low toxic against MDCK with TC_{50} values 299.59 and 395.86 µg/ml, respectively. Due to the uppermost cytotoxicity effect of EA against MDCK cells, the antiviral activity of EA was not tested. The DE exhibited the highest antiviral activity through the inhibition of H5N1 propagation with IC_{50} value 125 µg/ml and selective index 2.397 as compared to ME with IC_{50} value 1333.33 µg/ml and selective index 0.297 (Table 4). To the best of our knowledge, this is the first report regarding the antiviral effect of DE and ME extracts of *U. urens* against the highly pathogenic avian influenza H5N1 virus. By studying the mode of action of DE extract of *U. urens*, the results showed the potent virucidal effect of DE extract with 80% inhibition of viral propagation. The DE extract showed a limited effect on

viral replication and viral adsorption process with 25 and 45% inhibition of viral replication and viral adsorption, respectively. The antiviral effect of aqueous extract of *U. dioica* against the feline immunodeficiency virus (FIV) through the inhibition of FIV-induced syncytium formation was reported at different concentrations (0.5-1µg/ml) with maximum inhibition level of 84% (Manganelli *et al.,* 2005). The N-acetylglucosamine-specific lectin from *U. dioica* showed antiviral effect against human immunodeficiency virus type 1 (HIV-1) and type 2 (HIV2), cytomegalovirus (CMV), respiratory syncytial virus (RSV), and influenza A (H3N2) virus through cytopathic induction at an EC_{50} ranging from 0.3 to 9 µg/ml (Balzarini *et al.,* 1992). The potent anti-influenza (H5N1) effect of DE and ME extracts of *U. urens* in this study is in consensus with previous studies that reported the anti-influenza (H5N1) effect of different plant extracts (Gaafar *et al.,* 2015; Nagy *et al.,* 2018). The anti-influenza (H5N1) effect of DE and ME extracts of *U. urens* could be attributed to different phenolic compounds in ME extract and the high content of oxygenated and nitrogen-containing compounds such as terpenoids and alkaloids as presented in Tables 1 and 2. The recent study of Sadati *et al.* (2019) indicated the antiviral effect of different flavonoids: hispidulin, quercetin, luteolin, catechin, naringenin, kaempferol, vitexin, and chrysin against influenza virus (H1N1). These flavonoids may efficiently block the neuraminidase (NA), an enzyme able to break the glycoside bonds and empowers the viral invasion through the host cell membrane, active site as compared to oseltamivir (Sadati *et al.,* 2019).

Table 4. Cytotoxicity (TC_{50}, µg/ml) against Madin Darby Canine Kidney cells (MDCK), and H5N1 propagation inhibition (IC_{50}, µg/ml) of diethyl ether (DE), ethyl acetate (EA), and methanol (ME) extracts of *U. urens.*

Extract	Cytotoxicity (TC_{50}, µg/ml)	Viral inhibition (IC_{50}, µg/ml)	Selective index
DE	299.59	125	2.397
EA	54.49	NT	NT
ME	395.86	1333.33	0.297

TC_{50}: represents the concentration of extract causing 50% cell toxicity against Madin Darby Canine Kidney (MDCK) corresponding to the control (without extract); IC_{50}: represents the concentration of extract causing 50% inhibition on propagation of influenza virus H5N1corresponding to the control (without extract) by using Plaque reduction assay; NT: not tested due to the high toxicity against MDCK.

4. Conclusion

These results emphasize that bioactive ingredients-rich extracts from the Egyptian plant *Urtica urens* show a potent effect as antioxidant, anticancer, and anti-influenza (H5N1). These results support the positive effect of the successive extraction using polar gradient solvents on the chemical contents and biological activities of *U. urens.* The highest amounts of total phenolics, total flavonoids, and total tannins, along with higher concentrations of phenolic individuals were detected in EA extract compared to ME extract. The EA extract exhibited the highest DPPH, ABTS, and reducing power antioxidant activity. The DE and EA extracts have an efficient cytotoxicity effect against breast cancer cells (MCF-7) and colon cancer cells (HCT116). The DE exhibited the highest antiviral activity against the extremely pathogenic avian influenza H5N1 virus through the inhibition of H5N1 propagation, replication, and viral adsorption process. This study provides a new effective and bioactive chemical scaffolds from the Egyptian plant *U. urens* that might help in the development of new effective anti-cancer and antiviral bioactive compounds. Extra studies are required to isolate and identify the chemical structures of bioactive individuals responsible for the anticancer and anti-influenza effects and to elucidate the molecular mechanisms in both *in vitro* and *in vivo* models underlying the anticancer and anti-influenza (H5N1) activities.

Conflict of interest

The authors declare that they have no conflict of interest.

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GC-MS/MS Based Metabolite Profiling and Evaluation of Antimicrobial Properties of *Emblica officinalis* Leaves Extract.

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Abstract

Ever since, traditional plants and their products were consumed by human beings in diverse health matters to heal variety of infections. At the moment, in all over globe, the scientific attention and focuses are escalating with regard to phytomedicine. This study explores the value of secondary metabolites of *Emblica officinalis* leaves extract (EOLE) identified through GC-MS/MS profiling in different solvents against microbial strains. *In-vitro* antimicrobial activity was established by means of an agar well diffusion scheme at various loaded concentrations (25 µg/mL-1000 µg/mL). GC-MS/MS profiling confirmed the presence of major accredited compounds characterized as PDMTFM (Rt- 9.891 min and peak area-1.77%), P2,6-BDME (Rt-8.588 min, peak area - 100%), P2,5-BDME (Rt - 8.567 min, peak area - 36.78%) and BFTHTM (Rt-9.055 min, peak area - 7.87%) in tag plant. Gram positive bacteria; *Bacillus pumilis*, *Bacillus cereus*, *Staphylococcus pyogenous*, *Bacillus polymyxa* and Gram negative bacteria; *Vibrio cholera*, *Providencia alcalifaciens*, while fungal species; *Neurospora crassa*, *Trichoderma viridae*, *Aspergillus brasileinsis* and *Cladosporium oxysporum* were used during this study. Aqueous extract made known excellent antimicrobial activity than other selected extract. Inhibitory values of aqueous and methanolic extract were measured with reference to ciprofloxacin and amphotericin. Preliminary results of this study implied that EOLE could be considered as strong antimicrobial agent to treat human infectious diseases.

Keywords: Gas Chromatography, Amla leaves, Extraction techniques, Antimicrobial species

1. Introduction

Amla *(Emblica officinalis)* is a traditional herbal drug belonging to Euphorbiaceae family, usually recognised as "amalaki". Plant is small, consisting of spherical fruit with soft barks and leaves which are light green in colour, simple, subsessile, intimately situated by the side of branchlets. Leaves of *E. officinalis* are commonly a good source of secondary metabolites, acknowledged to be used in the management of a variety of maladies like over activity of thyroid, regulation of high blood glucose level, stimulation of antibodies formation, high level of bilirubin, bacterial disease like tuberculosis, dyspepsia as well decrease high cholesterol level (Ramesh *et al.,* 2015). Previous literature showed that the plant possesses several antimicrobial chemicals such as tannins, phenolics, flavanoids, alkaloids , tannins and carotenes (Banji *et al.,* 2018).

Asmawl *et al.,(*1993) and co-worker have reported that leaves of *E. officinalis* are rich in the polyhydroxy phenolics molecules like gallic acid, mallic acid, quercetine, chebulic acid and O-galloyl glucose respectively. The potential of antimicrobial activity of plant base drug has been recommended due to the presence of polyphenolics compounds. Researcher suggested a variety of techniques for the separation of phyto metabolites from the tissues of plants. While the selection of extraction method based on considerable issues specifically efficiency, robustness, resources outlay, running cost and ease of operation (Gulcin *et al.,* 2012).

In a previous study, several customary extraction methods such as Soxhlet, successive extraction, hot extraction, cold extraction, were recommended for extraction of herbal drugs. On the other hand, extraction by UAE is a recent procedure used to extract bioactive molecules from natural source. This is a trustworthy technique that condenses the release of hazardous pollutants by dropping the utilization of organic solvent and is relatively straightforward to operate. It compressed processing period, minimum chances of oxidation and decomposition of plant molecules and gave significant yield in the course of rapid extraction. Ultrasonic assisted extraction (UAE) may be introduced to isolate the critical medicinal constituents of *E. officinalis* (Shen and Shao, 2005). The adequate antimicrobial reports of *E. officinalis* leaves extract (EOLE) were available; on the other hand, inadequate information exist on the topic of mechanism and antimicrobial scope concerned with EOLE. In this

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regard, the intention of present study is to scrutinize the leaves constituents of *E. officinalis* by UAE technique in altered polarity solvents by means of GC-MS/MS profiling as well as to explore the *in vitro* activity of it against the microbial strains.

Nevertheless, herbal medicine remains the best choice of natural sources mining as antimicrobial remedies. In favor of our statement, there are still some microbial families for which antimicrobial activity of EOLE are less exposed in the earlier studies. There is, as a result, a positive call for the investigation of antimicrobial properties *E. officinalis* through sophisticated analytical techniques to encourage its use throughout the community. Consequently, we propose the title plant as potential candidates for further study in folk medicines to establish the better drugs against microbial disease.

2. Materials and methods

2.1. Equipment, chemicals and procurement of plant material assortment

Crude leaves of *Eumblica officinalis* were used in the proposed study. Plant material was purchased from community marketplace of Khari Bavli New Delhi-110006. The specimen (NBPGR/2017-2) was submitted and verified by National Herbarium of Cultivated Plants at Pusa campus, New Delhi-110012-India. Subsequently, leaves were unsoiled, washed down with water and after acclimatization extraction was executed on an ultrasonic system (Power flow-250 W and Frequency 35/60 kHz). GC-MS/MS profiling was deal with Agilent model 7000D (Triple Quad) system. Methanol was made available by Merck (Mumbai, India) and all other experimental chemicals and solvents were of analytical grade.

2.2. Plant extracts preparation by ultrasonic extraction technique

The dried leaves (5gm) were considered for extraction purpose through water and methanol (300 mL) by means of UAE, which connected with water bath, digital gauge and a device to control the temperature. The system was derived at a calculated frequency of 35/60 kHz, fixed ultrasonic input power of 250 W and experiment was repeated for thrice. To avoid excessive heat generation during ultrasonication, the system was carried out at moderate room temperature (25 ^{o}C ± 5). Active extract was recovered and dried by means of rotavapor to remove the trace amount of solvent completely in order to maintain its quality (Tonk *et al.*, 2006).

2.3. GC-MS/MS profiling

2.3.1. Samples preparation and GC-MS/MS background

For GC-MS/MS profiling, the sample was processed by transferring about 5 mg of extract in a graduated measuring flask and attended the volume up to 10 mL with various solvents (water and methanol); subsequently, the mixture was subjected to centrifugation at 6000 rpm for 5 min. Finally, the supernatants were filtered through 0.22 µm syringe filter and the experiment was performed with 1 mL of this solution.

The aqueous and methanolic extracts of *E. officinalis* leaves were submitted to GC-MS/MS (Triple Quadru pole/2010). The GC-MS/MS backgrounds were as follows: system installed with capillary column (5 % phenyl polysiloxane stationary phase x 30.0 m length × 0.25 µm film x 0.25 mm l.d), column oven temperature was set at 60 °C (fixed for 2 min), augmented at 15 °C per min to 270 °C (fixed for 2 min) and at the end fixed at 290 °C for 15 min. The flow rate of the carrier gas, Helium (99.9% purity) was maintained at a speed of 1.5 mL/min. The experiment was executed by loading 1.5 µL of the titled sample through a split ratio of 30: 1 and carried out in electron impact (EI) mode of -70 eV as ionization energy. The injector temperature was asserted at 260 °C (steady). Mass spectra was studied in scanning manner at 25-500 m/z. Sample spectra were analyzed and identified in contrast to spectral configuration documented by means of existing mass spectral database (NIST 14-MS database library).

2.4. Selection of microbial strains for study

2.4.1. Bacterial and .fungal strains

Design protocol is comprised of bacterial and fungal strains that are *Staphylococcus aureus, Bacillus subtilis, Bacillus megaterium, Bacillus pumilis, Bacillus cereus, Staphylococcus pyogenous* and *Bacillus polymyxa* (Gram-positive), *Pseudomonas aeruginosa, Escherichia coli, Vibrio cholera, Salmonella typhi, Shigella dysenteriae, Proteus mirabilis, Providencia alcalifaciens* (Gram negative bacteria) and *Aspergillus niger, Neurospora crassa, Penicillium chrysogenum, Trichoderma viridae, Aspergillus brasileinsis, Candida albicans* and *Cladosporium oxysporum* respectively belonged to fungi category respectively.

2.4.2. Evaluation of antimicrobial and antifungal activity

The antimicrobial assay of EOLE was executed through agar well diffusion method and it included different bacterial pathogens namely (Gram-positive), (Gram-negative) and fungal pathogens respectively (Faiza *et al*., 2012). Once the autoclaving process of media was completed, it kept on cool at 35 ^{o}C-50 ^{o}C. To validate the sterility, ready to use and cooled media was transferred in to glass petri plates of equal diameter (60 mm in diameter), after solidifying of agar media the crude plates were allow to incubate at 35 ^{o}C for 20–24 h. Approximately, 100 µL of the fresh inoculums of bacteria was uniformly dispersed on the smooth surface of solidified agar media while fungal strains were spread on potato dextrose agar media of each petri plate. The wells of identical size (6 mm diameter x 2 mm depth) were crafted by using sterile steel cork borer. About 0.1 mL of various concentrations of EOLE were poured in to the well with sterile glass syringe and set aside to diffuse completely for 2 h at room temperature. Immediately after diffusion, all the petri plate were allowed to incubate in incubator for 24-48 h at 37 ^{o}C used for bacteria strains and for 48-72 h at 30 ^{o}C for fungal strains, respectively. DMSO (0.05 mL) was utilized as a negative control for the study whereas ciprofloxacin (0.1 mg/mL) and amphotericin (0.1 mg/mL) employed as a positive control for bacterial and fungal strains, respectively. The antimicrobial results were documented by measuring the diameter (mm) of zone of inhibition with respect to each well. This study was performed every 3 times for each selected microbial strain.

3. Results

3.1. Selection of ultrasonic extraction technique

The classical extraction techniques are generally based on long exposure of heat reflex, skilled packing of plant material in thimble, demand for longer extraction period, unavoidable consumption and evaporation of solvent during experiment (Shen and Shao, 2005). To prevail these restriction of classical extraction techniques, a non classical ultrasound assisted extraction method has been considered good for the targeted plant sample with lower solvent evaporation and high percentage yield of aqueous (31.16 w/v) and methanolic (10.15 w/v) extracts respectively.

3.2. GC-MS/ MS profiling and spectrum characterization

The GC-MS spectrum of unknown phytomolecules of *E. officinallis* was interpreted in contrast with database of known molecules store in National Institute Standard and Technology (NIST) library. Figure.1 represents the distinct chromatogram of EOLE in targeted solvents. The plants derived bioactive constituents contribute a momentous role in various biomedical rationales. As revealed in Table 1, results of GC-MS profiling of EOLE comprise the retention time, molecular weight, molecular structure and peak area of active phytoconstituents. The major accredited compounds characterized were PDMTFM (Rt - 9.891 min and peak area - 1.77%), P2,6-BDME (Rt - 8.588 min, peak area - 100%), P2,5-BDME (Rt - 8.567 min, peak area - 36.78%) and BFTHTM (Rt - 9.055 min, peak area - 7.87%).

Table 1. Description and relative composition of bioactive constituents of *E. officinalis* leaves extract by GC-MS/MS.

E. officinalis leaves extract	Name of compound	Nature of compound	Formula	Mol. Wt. (g/mol)	Peaks	RT (min)	% Peak area
Aqueous extract	A. Phen-1,4-diol, 2,3-dimethyl 5trifluoromethy	Phenolic	$C_9H_9F_3O_2$	206	46	9.891	1.77
	B. Phenol, 2,6-bis (1,1-dimethylethyl)	Phenolic	$C_{14}H_{22}O$	206	4	8.588	100
Methanolic extract	C. Phenol, 2,5-bis (1,1-dimethylethyl)	Phenolic	$C_{14}H_{22}O$	206	16	8.567	36.78
	D.2(4H)Benzofuranone,5,6,7,7a-tetrahydro-4,4,7a-trimethyl	Heterocyclic Triterpene	$C_{11}H_{16}O_2$	180	23	9.055	7.87

RT: Retention Time; Mol.Wt.: Molecular weight

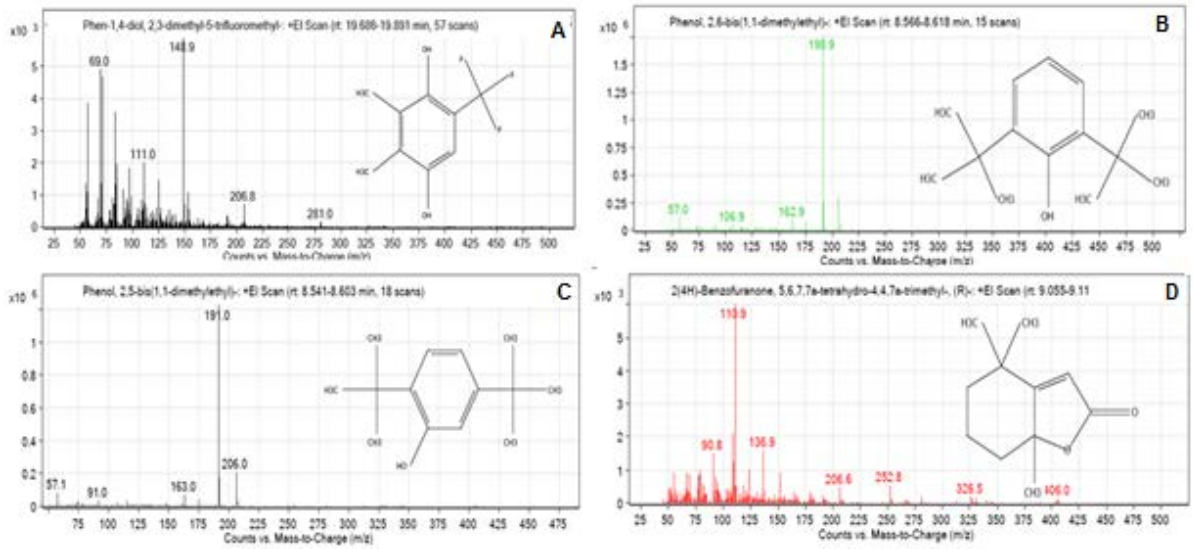

Figure 1. GC-MS/MS Chromatograms, structure and resultant mass peak of *E. officinalis* leaves.

3.3. Antimicrobial activity of designated plant extract

Antimicrobial prospective of different extracts of *E. officinalis* and standard antimicrobial drugs (CPF and AMP) against bacteria and fungi is represented in Table 2-7. Likewise, the zone of inhibition and MIC of standard antibiotics and EOLE were recorded at different concentration aligned with DMSO as negative control. Inhibitory effect of CPF was recorded at 25 µg/mL against all Gram positive and Gram negative bacteria and revealed highest zone of inhibition (15.05 ± 0.17mm) with *S. aureus* and *(*21.13 ± 0.18 mm) in contrast to aqueous and methanolic leaves extract of *E. officinalis* which exhibit no zone of inhibition at 25 µg/mL Table 2. Maximum resultant zone of inhibition against Gram positive bacteria; *B. pumilis* (7.20 ± 0.16mm), *B. megaterium*(5.30 ± 0.24), *B. cereus* (3.37 ± 0.20mm), *B. polymyxa* (3.27 ± 0.19) and Gram negative bacteria; *P. mirabilis* (4.40 ± 0.14mm), *V. Cholera* (4.27 ± 0.29mm), *S. typhi* (3.19 ± 026 mm), *P. aeruginosa* (2.00 ± 0.16) of aqueous extract were recorded at 50 µg/mL, while *B. subtilis*, *S. pyogenous*, *E. coli*, *S. dysenteriaea* and *P. alcalifaciens* respectively were not produced any zone of inhibition at this concentration Table 3. All reported bacteria gave observable zone of inhibition at the concentration (100 µg/mL) except *P. alcalifaciens.* It was observed that concentration ranging from (250 µg/mL–1000 µg/mL) of aqueous extract for the selected Gram positive bacteria and Gram negative bacteria exhibits good results while poor zone of inhibition with similar concentration was experienced with methanolic

extract Table 4. The results of our study proposed that aqueous and methanolic leaves extract of *E. officinalis* exhibited no antibacterial activity at the concentration (25 µg/mL). Only two Gram positive bacterial species *B. megaterium* (4.97 ± 0.29 mm), *B. pumilis* (4.83± 0.12 mm) and one Gram negative bacteria *V. cholera* (3.20 ± 0.18 mm) established that the concentration (50 µg/mL) was found appropriate for the activity although it showed no activity for *S. aureus, B. subtilis, B. cereus S. pyogenous, B. polymyxa, E. coli, P. aeruginosa, S. typhi, S. dysenteriae* and *P. alcalifacien.s.* Low inhibitory zone was experienced against all Gram positive and Gram negative bacteria, which required a very high concentration 250 µg/mL–1000 µg/mL excluding *P. alcalifaciens* to which no reaction was observed. The results of (Figure 2) confirmed that concentration (25µg/mL) of aqueous and methanolic extract was not found suitable for the antibacterial activity of the selected species. Although, no response was observed with two Gram positive bacteria; *B. subtilis, S. pyogenous* and three Gram negative bacteria; *E. coli, S. dysenteriaea* and *P. alcalifaciens* at concentration (50 µg/mL) of aqueous leaf extract, in another load five bacterial species *S. pyogenous, E. coli, S. typhi, S. dysenteriae, S. dysenteriae* exhibited no activity at (100 µg/mL) with methanolic extract of *E. officinalis*.

Anti fungal activity of standard amphotericin was not confirmed at (25 µg/mL), while concentration (250 µg/mL-1000 µg/mL) showed different level of zone of inhibitions Tables 5. Aqueous EOLE at (25 µg/mL-50 µg/mL) showed no clear zone of inhibition to *A. niger, N. crassa, P. chrysogenum, T. viridae, A. Brasileinsis, C. albicans* and *C. oxysporum*. Two fungal strain *A. Niger* (7.34 ± 0.9 mm) and *C. oxysporum* (3.35 ± 0.37mm) gave zone of inhibition at (100 µg/mL), while this dose was not satisfactory for other selected fungi. Purely, the growth of fungal strains was inhibited by EOLE at (250 µg/mL-1000 µg/mL). High inhibitory concentration of aqueous extract (100 µg/mL) were observed against *A. niger* (7.19 ± 0.90 mm) while *C. oxysporum* gave the lowest value (3.35 ± 0.17 mm) Tables 6. In comparison to aqueous extract of *E. officinalis*, no satisfactory antifungal activity was found with methanolic extract at (25 µg/mL–100 µg/mL). *A. niger* a single fungi merely gave zone of inhibition (5.27 ± 0.12) at 250 µg/mL. The results made known that a high concentration (500 µg/mL – 1000 µg/mL) of methanolic extract was needed to slow down the growth of concerned fungal species Tables 7. As shown in (Figure 3), the aqueous extract of *E. officinalis* best suited to slow down the fungal growth at a concentration (100 µg/mL) in association to methanolic extract which exhibited minimum antifungal action at higher concentration (250 µg/mL).

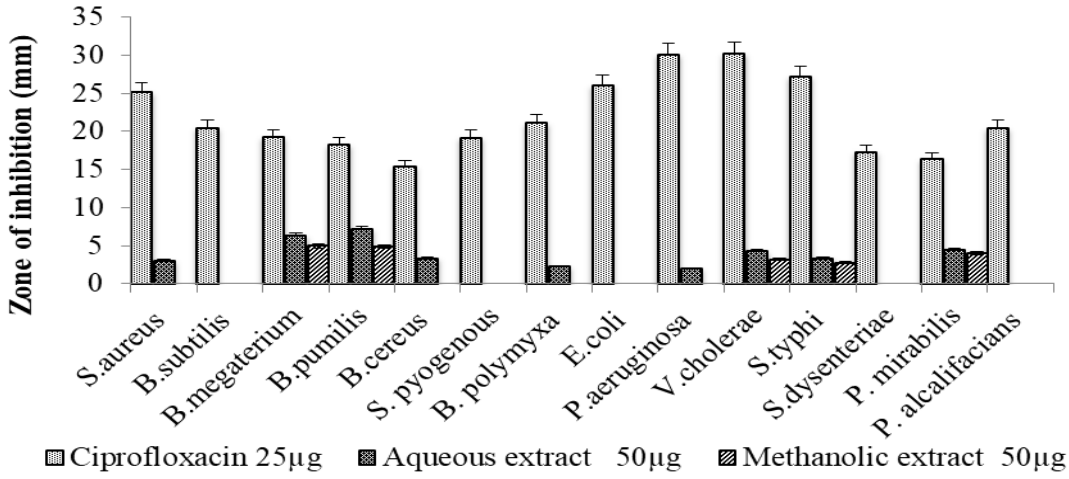

Figure 2. Ciprofloxacin and *E. officinalis* leaves extract which displayed widespread minimum inhibitory concentration (MIC) and name of bacteria against which they were active at different doses.

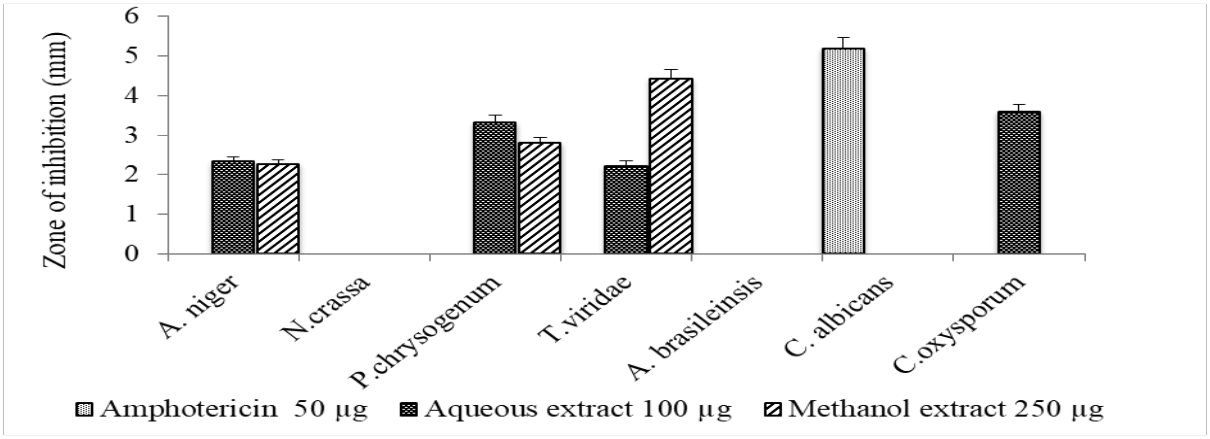

Figure 3. Amphotericin and *E. officinalis* leaves extracts which displayed widespread minimum inhibitory concentration (MIC) and the name of fungi against which they were active at different concentration.

Table 2. Antibacterial activity of standard (Ciprofloxacin) on Gram positive and Gram negative bacteria.

Types and name of Organisms		Concentration of standard (µg/mL) and Zone of inhibition (mm)[a] ± SD						
		25 µg	50 µg	100 µg	250 µg	500 µg	1000 µg	MIC µg
Gram positive	*S. aureus*	15.05 ± 0.17	15.63± 0.21	17.24 ± 0.11	17.33 ± 0.11	19.33 ± 0.31	*	25
	B. subtilis	9.14 ± 0.17	9.15 ± 0.21	9.27 ± 0.13	10.20 ± 0.13	10.96 ± 0.15	*	25
	B. megaterium	6.17 ± 0.15	8.23 ± 0.19	9.10 ± 0.15	9.75 ± 0.17	10.20 ± 0.12	*	25
	B. pumilis	13.10 ± 0.17	13.17 ± 0.15	14.12 ± 0.19	14.95 ± 0.01	15.10 ± 0.22	*	25
	B. cereus	11.51 ± 0.14	12.21 ± 0.23	12.55 ± 0.23	13.11 ± 0.29	14.37 ± 0.15	*	25
	S. pyogenous	9.25 ± 0.03	9.35 ± 0.24	9.47 ± 0.12	10.00 ± 0.17	10.15 ± 0.09	*	25
	B. polymyxa	8.03 ± 0.21	8.15 ± 0.10	8.75 ± 0.71	9.13 ± 0.11	9.0 ± 0.12	*	25
Gram negative	*E. coli*	17.15 ± 0.12	17.23 ± 0.03	17.35 ± 0.33	18.15 ± 0.22	18.37 ± 0.21	*	25
	P. aeruginosa	7.13 ± 0.05	7.41 ± 0.15	7.50 ± 0.17	8.25 ± 0.01	8.59 ± 0.71	*	25
	V. cholerae	10.19 ± 0.14	10.25 ± 0.09	10.75 ± 0.22	10.90 ± 0.43	11.05 ± 0.18	*	25
	S. typhi	9.13 ± 0.17	9.35 ± 0.21	9.53 ± 0.15	9.75 ± 0.11	10.00 ± 0.02	*	25
	S. dysenteriae	17.29 ± 0.23	18.42 ± 0.02	18.70 ± 0.04	18.90 ± 0.42	19.50 ± 0.03	*	25
	P. mirabilis	20.26 ± 0.21	20.39 ± 0.90	21.45 ± 0.19	21.50 ± 0.15	21.90 ± 0.01	*	25
	P. alcalifacians	19.43 ± 0.18	21.35 ± 0.07	21.50 ± 0.01	21.81 ± 0.22	22.25 ± 0.06	*	25
DMSO	Negative control	-	-	-	-	-	-	-

MIC µg: Minimum inhibitory concentration in microgram; SD: Standard deviation; DMSO: Dimethyl sulfoxide; CPF: Ciprofloxacin; Values are expressed as mean ± SD from three experiments ([a]n = 3); * Zones could not be measured due to margining.

Table 3. Antibacterial activity of aqueous leaves extract of *E. officinalis* on Gram positive and Gram negative bacteria.

Types and name of Organisms		Concentration of extract (µg/mL) and Zone of inhibition (mm)[a] ± SD						
		25 µg	50 µg	100 µg	250 µg	500 µg	1000 µg	MIC µg
Gram positive	*S. aureus*	NZI	3.00 ± 0.16	9.13 ± 0.26	11.50 ± 0.34	12.20 ± 0.16	12.63 ± 0.20	50
	B. subtilis	NZI	NZI	5.23 ± 0.21	7.77 ± 0.20	8.1.9 ± 0.08	9.63 ± 0.33	100
	B. megaterium	NZI	5.30 ± 0.24	6.47 ± 0.34	7.33 ± 0.24	8.83 ± 0.12	9.33 ± 0.25	50
	B. pumilis	NZI	7.20 ± 0.16	9.47 ± 0.29	10.50 ± 0.16	11.29 ± 0.25	12.87 ± 0.26	50
	B. cereus	NZI	3.37 ± 0.20	5.67 ± 0.21	6.87 ± 0.12	9.6 ± 0.21	11.23 ± 0.25	50
	S. pyogenous	NZI	NZI	3.13 ± 0.21	4.27 ± 0.20	8.63 ± 0.58	11.00 ± 0.62	100
	B. polymyxa	NZI	3.27 ± 0.19	6.13 ± 0.46	7.00 ± 0.29	7.40 ± 0.22	9.30 ± 0.29	50
Gram negative	*E. coli*	NZI	NZI	2.13 ± 0.25	4.57 ± 0.25	6.27 ± 0.25	7.50 ± 0.37	100
	P. aeruginosa	NZI	2.00 ± 0.16	5.90 ± 0.29	6.33 ± 0.17	7.03 ± 0.37	8.25 ± 0.17	50
	V. cholerae	NZI	4.27 ± 0.29	4.60 ± 0.29	4.97 ± 0.17	6.47 ± 0.33	7.07 ± 0.09	50
	S. typhi	NZI	3.19± 026	5.27 ± 0.30	6.13 ± 0.26	6.37 ± 0.33	9.30 ± 0.22	50
	S. dysenteriae	NZI	NZI	2.17 ± 0.17	5.43 ± 0.25	6.27 ± 0.21	10.25 ± 0.37	100
	P. mirabilis	NZI	4.40 ± 0.14	7.67 ± 0.29	9.87 ± 0.17	12.63 ± 0.43	13.43± 0.25	50
	P. alcalifacians	NZI	NZI	NZI	4.13 ± 0.20	6.10 ± 0.29	8.50 ± 0.46	250
DMSO	Negative control	-	-	-	-	-	-	-

MIC µg: Minimum inhibitory concentration expressed in microgram; NZI: No zone of inhibition; SD: Standard deviation, DMSO: Dimethyl sulfoxide; Data are expressed as mean ± SD as of three experiments ([a]n = 3)

Table 4. Antibacterial activity of methanolic leaves extract of *E. officinalis* on Gram positive and Gram negative bacteria.

Types and name of Organisms		Concentration of extract (µg/mL) and Zone of inhibition (mm)[a] ± SD						
		25 µg	50 µg	100 µg	250 µg	500 µg	1000 µg	MIC µg
Gram positive	*S. aureus*	NZI	NZI	7.83 ± 0.02	9.20 ± 0.43	11.13 ± 0.19	13.23 ± 0.16	100
	B. subtilis	NZI	NZI	4.80 ± 0.22	6.13 ± 0.09	9.90 ± 0.72	12.90 ± 0.70	100
	B. megaterium	NZI	4.97 ± 0.29	5.07 ± 0.25	6.33 ± 0.47	8.07 ± 0.25	9.37 ± 0.52	50
	B. pumilis	NZI	4.83 ± 0.12	5.40 ± 0.20	7.10 ± 0.05	7.90 ± 0.33	8.90 ± 0.08	50
	B. cereus	NZI	NZI	4.40 ± 0.06	4.90 ± 0.26	7.93 ± 0.38	9.93 ± 0.86	100
	S. pyogenous	NZI	NZI	NZI	2.60 ± 0.54	6.80 ± 0.59	10.20 ± 0.16	250
	B. polymyxa	NZI	NZI	4.90 ± 0.09	8.07 ± 0.17	11.33 ± 0.25	12.30 ± 0.24	100
Gram negative	*E. coli*	NZI	NZI	NZI	4.10 ± 0.30	6.23 ± 0.01	7.30 ± 0.20	250
	P. aeruginosa	NZI	NZI	5.07 ± 0.13	8.97 ± 0.12	10.50 ± 0.81	11.07 ± 0.25	100
	V. cholerae	NZI	3.20 ± 0.18	5.50 ± 0.05	9.03 ± 0.17	11.33 ± 0.47	13.93 ± 0.14	50
	S. typhi	NZI	NZ	NZ	2.65 ± 0.25	4.27 ± 0.38	5.96 ± 0.37	250
	S. dysenteriae	NZI	NZI	NZI	3.70 ± 0.24	5.73 ± 0.11	7.30 ± 0.22	250
	P. mirabilis	NZI	3.63 ± 0.33	3.97 ± 0.29	5.57 ± 0.01	8.63 ± 0.46	10.23 ± 0.01	50
	P. alcalifacians P. alcalifacians	NZI	NZI	NZI	NZI	3.57 ± 0.37	5.23 ± 0.71	500
DMSO	Negative control	-	-	-	-	-	-	-

MIC µg: Minimum inhibitory concentration expressed in microgram; NZI: No zone of inhibition; SD: Standard deviation, DMSO: Dimethyl sulfoxide; Data are expressed as mean ± SD as of three experiments ([a]n = 3)

Table 5. Antifungal activity of amphotericin.

Name of Organism	Concentration of AMT (µg/mL) and Zone of inhibition (mm)[a] ± SD						
	25 µg	50 µg	100 µg	250 µg	500 µg	1000 µg	MIC µg
A. niger	NZI	NZI	11.15 ± 0.01	13.30 ± 0.09	16.13 ± 0.19	17.11 ± 0.05	100
N. crassa	NZI	NZI	13.85 ± 0.01	15.54 ± 0.12	17.55 ± 0.03	18.57 ± 0.07	100
P. chrysogenum	NZI	NZI	NZI	13.57 ± 0.11	16.27 ± 0.17	16.51 ± 0.01	250
T. viridae	NZI	NZI	NZI	14.54 ± 0.15	17.52 ± 0.01	18.20 ± 0.14	250
A. brasileinsis	NZI	NZI	11.35 ± 0.01	12.51 ± 0.51	12.71 ± 0.05	14.17 ± 0.37	100
C. albicans	NZI	3.40 ± 0.05	5.17 ± 0.01	11.13 ± 0.05	14.13 ± 0.15	19.21 ± 0.05	50
C. oxysporum	NZI	NZI	NZI	9.15 ± 0.13	10.15 ± 0.24	15.25 ± 0.28	250
DMSO	-	-	-	-	-	-	-

MIC µg: Minimum inhibitory concentration expressed in microgram; NZI: No zone of inhibition; SD: Standard deviation, DMSO: Dimethyl sulfoxide; AMT: Amphotericin; Data are expressed as mean ± SD as of three experiments ([a]n = 3)

Table 6. Antifungal activity of aqueous leaves extract of *E. officinalis.*

Name of organism	Concentration of extract (µg/mL) and Zone of inhibition (mm)[a] ± SD						
	25 µg	50 µg	100 µg	250 µg	500 µg	1000 µg	MIC µg
A. niger	NZI	NZI	7.19 ± 0.9	9.05 ± 0.38	9.75 ± 0.21	10.53 ± 0.33	100
N. crassa	NZI	NZI	NZI	6.51 ± 0.16	8.54 ± 0.14	10.23 ± 0.29	250
P. chrysogenum	NZI	NZI	NZI	5.63 ± 0.17	7.27 ± 0.48	10.11 ± 0.12	250
T. viridae	NZI	NZI	NZI	7.61 ± 0.22	8.67 ± 0.29	11.27 ± 0.20	250
A. brasileinsis	NZI	NZI	NZI	5.80 ± 0.33	6.87 ± 0.20	9.45 ± 0.71	250
C. albicans	NZI	NZI	NZI	6.85 ± 0.25	7.1 ± 0.30	8..35 ± 0.65	250
C. oxysporum	NZI	NZI	3.35 ± 0.17	5.67 ± 0.17	6.21 ± 0.31	9.80 ± 0.12	100
DMSO	-	-	-	-	-	-	-

MIC µg: Minimum inhibitory concentration expressed in microgram; NZI: No zone of inhibition; SD: Standard deviation, DMSO: Dimethyl sulfoxide; Data are expressed as mean ± SD as of three experiments ([a]n = 3)

Table 7. Antifungal activity of methanolic leaves extract of *E. officinalis.*

Name of organisms	Concentration of extract (µg/mL) and Zone of inhibition (mm)[a] ± SD						
	25 µg	50 µg	100 µg	250 µg	500 µg	1000 µg	MIC µg
A. niger	NZI	NZI	NZI	5.27 ± 0.12	6.80 ± 0.41	8.89 ± 0.37	250
N. crassa	NZI	NZI	NZI	NZI	4.50 ± 0.41	7.43 ± 0.33	500
P. chrysogenum	NZI	NZI	NZI	NZI	5.20 ± 0.16	8.37 ± 0.32	500
T. viridae	NZI	NZI	NZI	NZI	4.39 ± 0.26	7..25 ± 0.20	500
A. brasileinsis	NZI	NZI	NZI	NZI	6.45 ± 0.41	8.43 ± 0.45	500
C. albicans	NZI	NZI	NZI	NZI	5.37 ± 0.12	7.97 ± 0.24	500
C. oxysporum	NZI	NZI	NZI	NZI	5.53 ± 0.17	7.13 ± 0.20	500
DMSO	-	-	-	-	-	-	-

MIC µg: Minimum inhibitory concentration expressed in microgram; NZI: No zone of inhibition; SD: Standard deviation, DMSO: Dimethyl sulfoxide; Data are expressed as mean ± SD as of three experiments ([a]n = 3)

4. Discussion

4.1. Ultrasonic extraction technique

Plant and plantconstituents give their important contribution to the wellbeing from the time immortal. Plant base metabolites are procured by various traditional extraction techniques namely filtration, maceration, decoction and soxhlet extraction while non-traditional modes of extractions are microwave-assisted extraction, supercritical fluid extraction and ultrasonic-assisted extraction. Ultrasonic extraction technique is an easy, straightforward, uncomplicated and unsophisticated technique. The competence of phyto extraction depends on various issues, including temperament of plant base metabolites, method of separation, particle size of crude drug to be extracted, extraction period, temperature of unit, pH of solvent, compatibility of solute to solvent and solvent polarity (Gonbad *et al*., 2015). In an existing study, ultra sonic extraction technique was used for the extraction of plant constituents from the leaves of *E. officinalis*, and their extractive yield was calculated. High extraction yield was achieved with water 3.7 g while methanol yielded 3.0 g respectively. High extractive value showed better extractive efficiency of aqueous solvent than methanol and this result was established to be analogous with the existing study . The reported technique is a proficient that needed a very short duration of run period and consume a smaller amount solvent. It condenses the load of harmful toxicants of organic solvent to the extract; the technique is robust, effective, precise and relatively straightforward to manage. The prospects of solvent oxidation, crumbling and rotting of plant metabolite are very less to this extraction technique (Khaled *et al.,* 2018). Due to these qualities, UAE attended more attention than other traditional extraction techniques, to fulfil the extraction demand of the plant metabolite. The proposed mechanism behind robustness of UAE process extraction efficiency was increased in surface areas, speed of mass transfer and assembly of several bubbles which generate significant interior heat and load on tissues causing cell destruction of plant tissues (Nostro *et al.,* 2000).

4.2. Characterization of potential compounds by GC-MS/MS

Characterization of phytoconstituents has been done by GC-MS/MS technique, a versatile analytical tool which is repeatedly the choice of many researchers, adopted due to its robustness. Different types of phenolics compound are characterized in plant extracts; in this view Dharni *et al.,* 2014, proposed 2,4-Di-tert-butylphenol (2,4-DTBP) as a potent antibacterial and antifungal natural compound characterised by GC-MS analysis. In an additional report, Sangmanee and Hongpattarakere (2014) suggested that low concentration (100 µg/mL) of (2,4-DTBP) which was detected by GC–MS profiling slows down the speed of spore germination of *F. oxysporum.* Padmavathi *et al.,* 2014, present in their study the antibacterial activity of *S. marcescens*, a Gram-negative bacteria was inhibited by polyphenolic constituents 2,4-DTBP and PBDME. The proposed findings are completely synchronize by means of the earlier facts, in which the phenolic compounds, 2,4-DTBP and PBDME showed good antioxidant aptitude because of their ability to forage free radicals and active oxygen species. Recently, an active constituent BFTHTM was fractioned from the leaves of *Azadirachta indica*, used as an analgesic, antiinflamatory, antidiabetic, antimicrobial agent (Rangel-Sanchez *et al.,* 2014). P2,6-BDME a polyphenolic compound, has also reduced the microbial load and slowed down the spore germination belonging to Enterobacteriaceae family (Mujeeb *et al.,* 2014). Our results corroborate with an earlier study and proposed that certain volatile organic composite P2,6-BDME, BFTHTM, PDMTFM present in EOLE established an antifungal property of EOLE (Yahiaoui *et al.,* 2017).

4.3. Antimicrobial activity

Today, a number of synthetic antibacterial and antifungal drugs are available to the medical field; however, their casual and regular use fixed a drug resistance aptitude (Anurag *et al.,* 2017). To overcome this menace, there is a continuous need to find the appropriate solution. In our findings, we proposed plant source drugs, as the available modern drugs responsible for a number of side effects in an individual. To this fact, we tested antimicrobial activity of EOLE and the growth of all microbes were successfully inhibited by aqueous extract in comparison to methanolic extract. *B. cereus, B. pumilis*

and P. mirabilis causing health issues to a millions of general public all over the globe while data of previous four decades raise the issues of subclinical urinary tract disease by *P. mirabilis* (Armbruster and Mobley, 2012). One more survey publicized that antibodies against proteus microbes were augmented considerably in patients and there are fewer chances for *P. vulgaris* to be isolated commonly in the urine of patients in contrast to other selected microorganism (Drzewiecka, 2016). Consequently, the use of aqueous EOLE may be advantageous towards these diseases sourcing microorganism. The inhibitory pattern of the microorganism showed a discrepancy with the nature of solvent extracts and this may possibly be due to discrepancy in the extraction procedures and microorganism to be tested (Mujeeb *et al.,* 2014). The antibacterial action of the crude extract of EOLE was tested against CPF (positive control) and DMSO as a negative control at different dilution. CPF inhibited the growth of all microbes at 25 µg/mL. To ascertain the antifungal activity of EOLE, AMT was used as standard and the value of inhibitory zone was recorded against DMSO. A high concentration (500 µg/mL–1000 µg/mL) of methanolic EOLE was needed to inhibit the augmentation of all diseases causing fungi whereas the study proposed that the multiplications of the fungus was greatly reduced by means of aqueous extract of *E. officinalis* at low dose (100 µg/mL) while methanolic extract demonstrated nominal antifungal action at higher dose (250 µg/mL). The antifungal action of extracts might be due to the additive outcome of different active phytoconstituents in both extracts. To support our result and mechanism, EOLE was subjected to GC-MS/MS profiling which revealed the existence of PDMTFM, P2,6-BDME and P2,5-BDME 2,4-Di-tert-butyl phenol (2,4-DTBP) which belongs to terpenes and phenolics categories, and predominantly mono and sesquiterpenes are able to inhibit the growth of bacteria, fungi, virus and protozoan (Habtemariam and Gray *et al.,* 1993). The greatest antibacterial and antifungal activity of aqueous extract against all the targeted microbes may possibly be due to high solubility of plant metabolites extracted in the solvent (Kumar and Pandey, 2012). These phytoconstituents, conceivably, may inhibit microbial acceleration efficiently by binding to their cell membrane (Kai *et al.,* 2007). In support of this, the antimicrobial activity of aqueous EOLE can be explained due to the presence of secondary plant metabolites, such as tannins, terpenoids, alkaloids and flavonoids (Siddiqua *et al.,* 2015). Earlier results have assured that the potential antimicrobial properties of EOLE can be most probably associated with the existence of secondary plant metabolites and the collective effect of these compounds absorption onto the cell surface causing its distraction and cell contents leakage and the production of hydroperoxides by polyphenolic compounds (Brul and Coote, 1999). These outcomes validate the healing property of *E. officinalis* leaf extracts. However, the majority of the experiments have appraised antimicrobial and antifungal properties of *E. officinalis* fruits extracts and some degree of magnitude is specified to the extract of other part of the plant

5. Conclusion

It can be concluded that results of present report, focused on the activity of phytoconstituents of aqueous and methanolic extract to their different polyphenolics compounds. The significant inhibitory role of targeted drug against a variety of microbial strains was primarily due to the occurrence of secondary metabolites. GC-MS profiling confirmed the occurrence of a number of active constituents belonging to flavanoids and phenolics compounds. EOLE showed a wide spectrum of biological activity against pathogenic Gram Positive bacteria and Gram negative bacteria as well as fungal species. These conclusions give a reason of choice for antimicrobial study in order to monitor the activity of the extract of traditional herbal medicine *E. officinalis* and their products that may hold rich contents of phenolic compounds.

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Effect of *Pleurotus ostreatus* Extract on Epidermal Growth Factor Receptor Expression during Healing of Aspirin-induced Peptic Ulcer in Male Rats

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Abstract

Peptic ulcer is an international problem in the last two centuries, including gastric and duodenal ulcers. The great majority of peptic ulcer disease is due to peptic inflammation caused by the infection of *Helicobacter pylori* and non-steroidal anti-inflammatory medicines. The purpose of this study was to investigate the healing role of *P.ostreatus* extract on peptic ulcers throughout the detection effect of *P. ostreatus* extract on epidermal growth factor receptor (EGFR) expression intensity during ulcer healing. In this study, 60 male rats of *Rattus norvegicus* were used; rats were divided into negative control group (C) given standard diet and distilled water only along study period; the remaining rats were treated by aspirin (100 mg/kg body weight) for induction of ulcer by oral dosage for one week, then these animals were divided into T1 (positive control group or aspirin ulcerated group which developed peptic ulcer through oral dosage for one week and remained without treatment for 20 day), T2 (group treated with omeprazole for 20 days), T3 (group treated with alcoholic extract of *P.ostreatus* for 20 days), T4 (group treated with polysaccharide of *P. ostreatus* for 20 days) and T5 (group with chitin and chitosan of *P. ostreatus* for 20 days). The immunohistochemical technique was used to identify the gene expression of EGFR. The results of this study showed there is a significant increase in the mean of EGFR expression intensity in the stomach and duodenal ulcer treated by mushroom extract and omeprazole in comparison with the untreated ulcer in positive control group T1 ($p<0.05$) at 10 day and 20 days. Moreover, the highest means of expression of EGFR intensity were recorded in the case of T3 followed by the treatment T4 at 10 and 20 days respectively. In conclusion, alcoholic extract of *P. ostrateus* has a significant role in peptic ulcer healing throughout the increasing expression of EGFR intensity.

Keywords: *P.ostrateus*, EGFR, peptic ulcer, polysaccharide, chitin&chitosan.

1. INTRODUCTION

Peptic ulcers, including gastric and duodenal ulcers, have long been classified as one of the most common diseases affecting humans in general, particularly young people (Kim *et al*.,2014). *H. pylori* infection and nonsteroidal anti-inflammatory drugs became the major causes of peptic ulcer (Mohan, 2018). Aspirin is an effective non-steroidal anti-inflammatory drug (NSAID) used to treat rheumatoid arthritis and related diseases and avoid thrombotic cardiovascular diseases. A major problem is a gastric ulcer associated with aspirin use. (Yin *et al*., 2007)

The process of ulcer healing involves growth factors that appear to play a crucial role in the stimulation of reconstruction of damaged mucosal structures. Among these factors, the most relevant are epidermal growth factor (EGF) (Kangwan *et al*.,2014). EGF ،a simple 53-amino acid polypeptide, is a cell-surface protein primarily responsible for increased cell proliferation at the mucosa of the ulcer margin and re-epithelialization; hence, its receptors are expressed as gastric glands dilate and the lining epithelial cells de-differentiate. It is mainly produced in salivary glands but can also be found in duodenal and pancreatic juice (Alese *et al.*,2017).

Epidermal growth factor was implicated in ulcer protection and ulcer healing. Through interaction with its cell surface receptor (EGFR). studies found that EGFR showed good digestive tract mucosal protection. Its expression in normal gastric mucosa is few, while gastrointestinal damage can increase its level (Aupperlee *et al*.,2012). EGFR can be activated by the binding of its ligands, including EGF, transforming growth factor-α, heparin-binding EGF-like growth factor, amphiregulin, betacellulin and epiregulin (Berasain and Avila 2014)

The effects of these growth factors are pleiotropic, ranging from the induction of DNA synthesis and changes in cell adhesion and motility to the stimulation of the differentiated cell function. In particular, EGF as well as TGF-α play important roles in the proliferation and differentiation of mucosal cells in the gastrointestinal tract, including the stomach (Brandl *et al*., 2010). More than 50% of all modern clinical drugs for peptic ulcers are of natural product origin and play a major role in the pharmaceutical industry's drug development programs

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(Lee *et al*., 2015). Mushrooms have recently become attractive as a functional food and as a source for new drug production; it has been stated that the functions of the immune system are activated by polysaccharides from the fruiting body or mycelium from a wide variety of edible mushrooms, fungi, and bacteria. Polysaccharides, including β-glucans, are now widely recognized as modifications of biological responses (Wilbers *et al*., 2016; Padilha *et al*., 2009). Dilfy *et al*., (2020) showed that chemical analysis of *P.ostreatus* showed that this mushrooms are an important source of various amino acids, vitamins, sugars, and minerals that considered important to body functions. Patel *et al*., (2012) stated that *P. ostreatus* had multiple active metabolites used as an anti-(aging, cholesterol, hyperglycemia, hypotensive, inflammatory, immunodeficient, microbial, mutagenic and neoplastic), metabolites besides as a defense and immunomodulation agent, which acts as an antibodies and tumor for the prevention and treatment of various human conditions. Present study aimed to use mushroom extract of Iraq cultivated *P.ostreatus* as success treatment for peptic ulcer throughout detection of the therapeutic effect of this mushrooms on EGFR during ulcer healing.

2. MATERIALS AND METHODS

2.1. Preparation of mushroom extracts

The fresh fruiting bodies of *Pleurotus ostreatus* were supplied from the Ministry of Science and Technology-Directorate of Agricultural Research-Baghdad. The first step in the preparation of mushroom extracts is cutting fresh fruiting bodies of mushroom (*P. ostreatus*) with a sharp knife into small parts. The parts were well air-dried for one week and kept in a night oven at a temperature of 40 ℃ to prevent rot. The air-dried mushroom sample was blended into powder using a blender. The crushed biomass (100 g) was placed in 400 ml of absolute methanol and incubated for 48 hours at 200 rpm. And the temperature is 37 ℃. To remove the biomass, the suspension was filtered by Whatman filter paper No.2. The supernatant was concentrated under lower pressure in a rotary evaporator at 50 ℃. The results were kept at 4 ℃ in the dried biomass (Jedinak and Sliva, 2008). Extraction of polysaccharide was prepared according to the method of (Gaur *et al*.2016), and extraction of chitin &chitosan according to the method of (Erdogan *et al*.,2017).

2.2. 2.2 Aspirin preparation

Aspirin was used for the induction of gastrointestinal ulcers in all experimental animals except the negative control group. To obtain the required dose (100 mg/kg b.w), a solution was prepared by dissolving 1 g of aspirin powder in 100 ml of 5% Carboxymethylcellulose to be aspirin concentration 10 mg /ml and given 1 ml / 100 g b.w (Seo *et al*.,2012).

2.3. Omeprazole preparation

Omeprazole is a standard drug, utilized in the treatment of ulcers developed in animals. To obtain the required dose (20 mg/kg BW), a Stock solution of this drug was prepared via dissolving of Omeprazole (0.2 g) in 100 ml of distilled water and given 1 ml /100 g body weight (Cavallini *et al.*,2006).

2.4. Animals

In this study, 60 male rats of *Rattus norvegicus* were used; rats were divided into negative control group (C) which given the standard diet and distilled water only along study period. The remaining rats are treated by aspirin (100mg/kg body weight) for induction of ulcer by oral dosage for one week, then these animals were divided into T1(positive control group or aspirin ulcerated group which developed peptic ulcer through oral dosage for one week and remained without treatment for 10 and 20 days), T2 (group ulcerated males rats were treated with omeprazole), T3 (group ulcerated males rats were treated with alcoholic extract of *P. ostreatus* for 10 and 20 days), T4(group ulcerated males rats were treated with polysaccharide of *P.ostreatus* for 10 and 20 days) and T5 (group ulcerated males rats were treated with chitin and chitosan of *P. ostreatus* for 10 and 20 days). Each group was anesthetized after the end of 10 days and 20 days and samples were taken directly.

2.5. Immunohistochemistry for detection of EGFR protein in peptic ulcer

Immunohistochemistry is a technique used for detecting cellular or tissue components (antigens) through antigen-antibody interactions. EGFR antibody and ABC staining system (Poly-HRP Anti Rabbit/Mouse IgG detection) were provided by Elab science. The procedure of immunohistochemistry for detection EGFR intensity in stomach and duodenum tissue was performed according to company manufacture. When the binding happens between antigen and antibody (Ag-Ab), a colored histochemical reaction product can be examined by light microscopy. Quantification of the EGFR expression (mm2) area by using Image-J software the photographs of the stomach and duodenum were digitalized and converted to binary images through grayscale imaging (Khan, 2004)

*2.6. **Statistical analysis***

The statistical significance of differences between groups was evaluated using single-way ANOVA, and the $p<0.05$ value was considered to be important. The sum of all values is expressed as a standard deviation in the mean.

3. Results

The results of this study showed a significant increase in the intensity of epidermal growth factor receptor (EGFR) expression in the treated stomach and duodenal ulcer compared with the untreated ulcer in the positive control group (T1) ($p<0.05$) during experimental period (**Table 1).** The results showed that EGFR expression intensity in the positive control group (T1) was extremely weak immuno-reactive in both stomach and duodenum tissue as (355 ±16 and 288±42) at 10 days and (409±20 and 315±66) at 20 days respectively.

The results revealed a significant increase in the mean of epidermal growth factor receptor (EGFR) expression intensity in treated stomach ulcer, and the highest means of expression intensity were recorded in groups (T3, T2 and T4) which were (3841±201, 2688 ±114 and 2656±138 respectively) at 10 days period. The intensity of EGFR expression in these groups decreases at 20 days with means (2809±113, 2301±106.5 and 1889±120 respectively). The lowest means of EGFR expression

intensity in stomach ulcers were recorded in the case of T5 which were 1197±99.65 and 1343±100 at 10 and 20 days respectively.

Statistically, all treatments (T2, T3, T4, T5) differed significantly in the mean of expression intensity of epidermal growth factor receptor (EGFR) when compared with the negative (C) and positive control group (T1) and in EGFR expression intensity between stomach and duodenum tissue at 10 and 20 day (**Table 2).**

Table 1. Effect mushroom extract and compounds on EGFR expression of gastrointestinal ulcer at 10 days and 20 day

Experimental Groups	Stomach EGFR expression intensity		Duodenal EGFR expression intensity	
	at 10 day	at 20 day	at 10 day	at 20 day
	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD
C	306±19	310±33.7	302.±27.8	336±28
T1	355±16	409±20	288±42	315±66
T2	2688±114**	2301±106.5**	656±58**	641±23.4**
T3	3841±201**	2809±113**	690±49.5**	668±30**
T4	2656±138**	1889±120**	567±17.5**	413±11**
T5	1197±99.65**	1343±100**	333±24.3**	403±19.8**

- SD= Standard Deviation; **= significant association in compared with T1 (P<0.05)

Table 2. Compared EGFR expression between treated stomach ulcer and treated duodenal ulcer at 10 days and 20 day

Experimental Groups	at 10 day			at 20 day		
	Stomach EGFR intensity	Duodenal EGFR intensity	P-value	Stomach EGFR intensity	Duodenal EGFR intensity	P-value
	Mean ± SD	Mean ± SD		Mean ± SD	Mean ± SD	
C	306±19	302.±27.8	0.436	310±33.7	336±28	0.711
T1	355±16	288±42	0.054	409±20	315±66	0.651
T2	2688±114**	656±58**	<0.0001[S]	2301±106.5**	641±23.4**	<0.0001[S]
T3	3841±201**	690±49.5**	<0.0001[S]	2809±113**	668±30**	<0.0001[S]
T4	2656±138**	567±17.5**	<0.0001[S]	1889±120**	413±11**	<0.0001[S]
T5	1197±99.65**	333±24.3**	<0.0001[S]	1343±100**	403±19.8**	<0.0001[S]

- SD= Standard Deviation ; S= significant association (P<0.05)

The result also demonstrated that the EGFR expression intensity in stomach tissue reached a peak at 10 days then decreased in the second period (20 days) in groups (T2, T3, and T4), whereas EGFR expression intensity in the group (T5) was still increasing during the time of treatment until 20 days as shown in **Figure (1).**

Similar results were observed in the duodenal ulcer that expression intensity increased at 10 days in groups (T2, T3, and T4) then decreased at 20 days. The intensity of expression in the group (T5) continued to increase with time of treatment as shown in **Figure (2).**

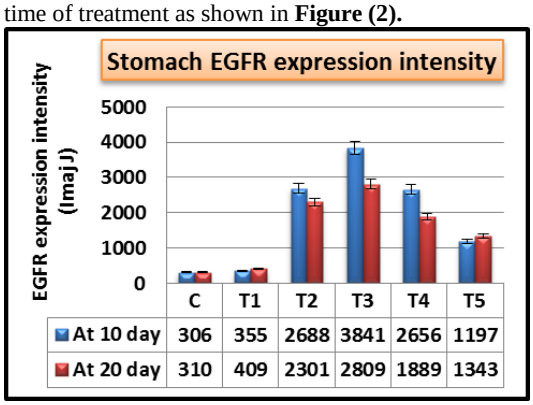

Figure 1. Effect mushroom extract and compounds on EGFR expression intensity of stomach ulcer after 10 days and 20 day.

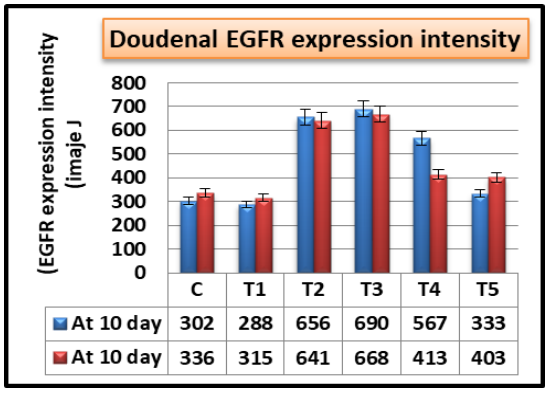

Figure 2. Effect mushroom extract and compounds on EGFR expression intensity of duodenal ulcer at 10 days and 20 day.

Anatomically, the study had been showing an increasing intensity of EGFR expression in the treated stomach and duodenal ulcer, and this increasing was related with clear mucosal healing especially in groups T3 at 10 and 20 days of the experiment (Figures 3,4)

The highest means of EGFR expression intensity in peptic ulcers recorded in T3 group may be due to the effect of the bioactive compounds of mushroom extract which induce the production of EGFR, and this may be related with the healing of ulcer and scar formation.

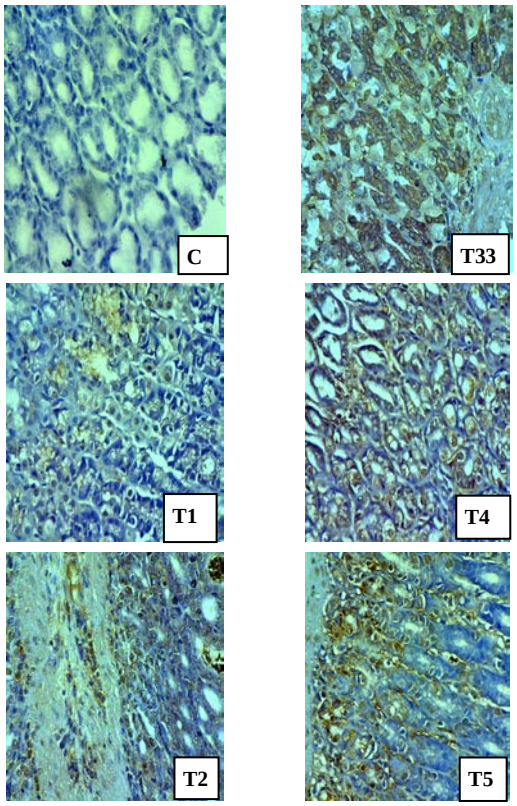

Figure 3. IHC staining for EGFR of stomach tissue at 10 days in treated groups (C: Control, T1: aspirin group,T2:omeprazole treated group,T3:*P.ostrateus* treated group,T4:*P.ostrateus* polysaccharide treated group and T5:chitin&chitosan *P.ostreateus* treated group) (X400).

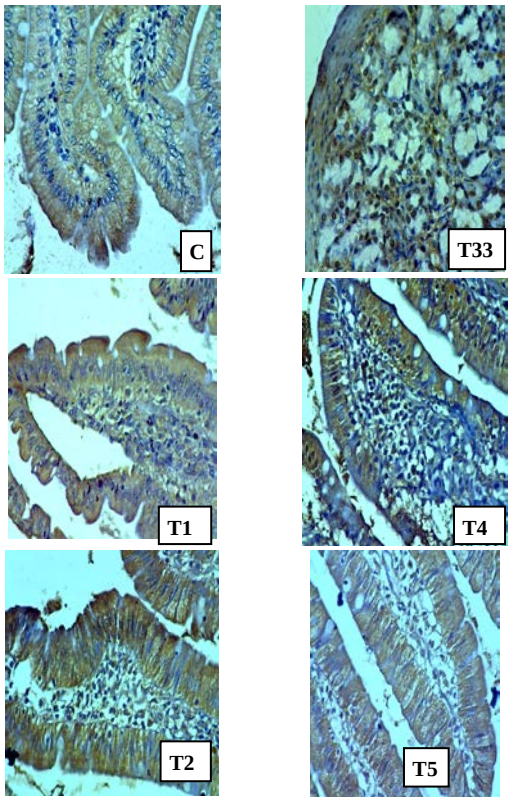

Figure 5. IHC staining for EGFR of duodenal tissue at 10 days in treated groups (C:Control,T1:aspirin group,T2:omeprazole treated group,T3:*P.ostrateus* treated group,T4:*P.ostrateus* polysaccharide treated group and T5:chitin&chitosan *P.ostreateus* treated group) (X400).

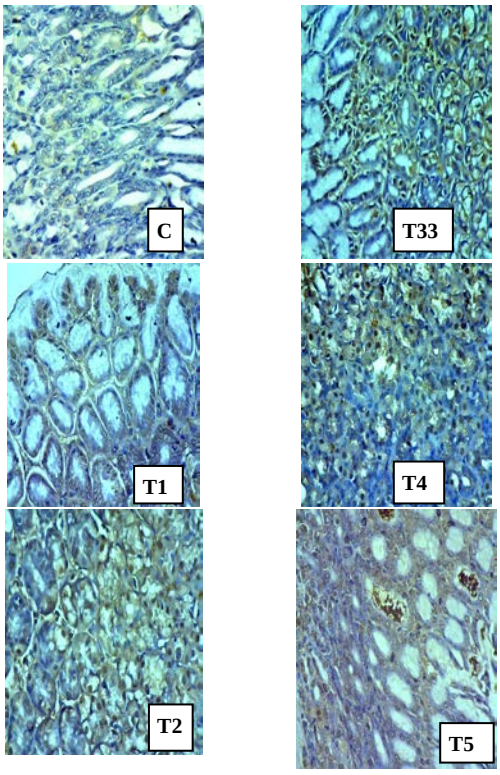

Figure 4. IHC staining for EGFR of stomach tissue at 20 days in treated groups (C: Control, T1:aspirin group,T2:omeprazole treated group,T3:*P.ostrateus* treated group,T4:*P.ostrateus* polysaccharide treated group and T5:chitin&chitosan *P.ostreateus* treated group) (X400).

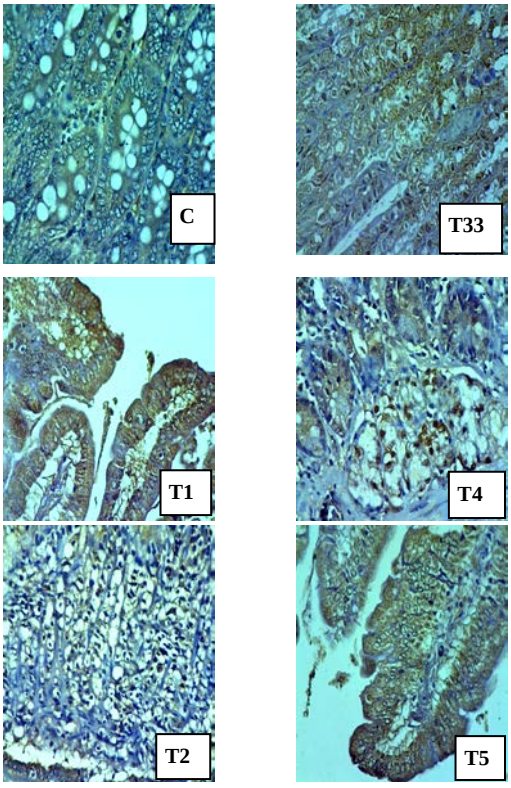

Figure 6. IHC staining for EGFR of duodenal tissue at 20 days in treated groups (C:Control,T1:aspirin group,T2:omeprazole treated group,T3:*P.ostrateus* treated group, T4:*P.ostrateus* polysaccharide treated group and T5:chitin&chitosan *P.ostreateus* treated group) (X400).

4. Discussion

Ulcer healing is an active and complex process that requires the coordinated interaction of various cellular and connective tissue components. During ulcer healing, processes including mucosal cell migration, proliferation and biochemical events are modulated by various growth factors, transcription factors and cytokines (Tarnawski,2005)

Many growth factors including Epidermal Growth Factor (EGF) contribute to the mechanism of ulcer healing as a result of the stimulatory effect on the process of mucosal repair, cell migration and proliferation as well as angiogenesis. The mechanism through which EGF initiate tissue healing is that when EGF binds to EGFR on the cell surface it will induce the activity of internal protein called tyrosine-kinase. This protein has triggered a signal transduction cascade that leads to a range of biochemical changes inside the cell, such as . intracellular calcium, glycolysis and protein biosynthesis and the expression of certain genes, including the epidermal growth factor receptor gene, which eventually leads to DNA synthesis and cell proliferation (Gupta *et al.*, 2019).

In this study, immunohistochemistry was used to assay the EGFR expression in aspirin induced gastroduodenal ulcer in male rat; the results showed a significantly reduced expression of EGFR in the aspirin group when compared with the treated groups. This may be due to suppression of endogenous NO synthesis that leads to delayed ulcer healing and high doses of aspirin impair normal NO activity, thereby suppressing the expression of growth factors (EGFR) in ulcer progression (Lanas *et al.*, 2000)

In normal control group (C), the expression of EGFR disappeared or appeared weakly only in proliferative zone and some of the parietal cells and this result are compatible with study of (Choi *et al.*, 2008) who found that EGFR was mainly localized in some mucous neck cells of the proliferation zone in normal gastric mucosa during immunohistochemistry examination.

The number of EGFR positive cells in the treatment groups significantly increased when compared with control and aspirin only groups throughout the experimental period. Research indicated that EGFR was closely related to the healing of impaired gastric mucosa and was of great importance in gastric mucosal protection (Alese *et al*.,2017)

Overexpression of EGFR has occurred during time of ulcer healing, and this is in agreement with the study of Choi, *et al*. (2014) who found significantly increased EGFR expression at 12 h, 24 h and 3 days after induction ulcer in the rat by using acetic acid. Szabo and Vincze,(2000) also recorded high expression of EGFR during early period of ulcer healing.

In this study, the expression of EGFR was increased in mushroom extract or compound treated groups; this might be suggestive of another probable mechanism by which the mushroom might have facilitated experimentally induced peptic ulcer healing. Moreover, the present study is the first to investigate the effect of extract of *P.ostrateus* on the expression of EGFR in peptic ulcer.

In the present study, the highest expression of EGFR in gastrointestinal ulcers was in group T3 and T6, treated by extract of *P.ostrateus*. Chemical composition of these fungi may be a good stimulator for the production of EGF, which can improve the quality of ulcer healing, and promote ulcer healing and tissue repair via binding with EGFR. Currently, EGFR is considered as an important evaluation indicator of the quality of ulcer healing (Faure and Lafont, 2013)

Lam *et al.* (2012) noticed that the extract of white button mushroom has an effective role in skin wound healing process which stimulates the proliferation of epidermal cells and suppresses cell death and inflammatory reaction. Study conducted by Hanawi *et al*.(2020) showed that the mushroom has curative effect on aspirin damaged tissue in stomach ulcer and preserved on normalcy histological architecture of stomach. The present results are comparable with Chen *et al.* (2016) who showed the healing of acute gastric lesions and chronic gastric ulcers is accompanied by an increased expression of EGFR in the ulcer area. Yan, *et al.* (2019) reported the correlation between the levels of epidermal growth factor EGF and the healing of the acute gastric mucosal injury in rats.

Fungal metabolites were known to produce a large and varied number of biologically active compounds that not only stimulate the immune system but also modulate specific cellular responses by interfering with particular signal transduction, antiulcer and a broad spectrum of healing activities such as asthmatic, anti-inflammatory, anti-obesity, anti-oxidative and anti-tumor (Yap *et al*., 2018; Samsudin and Abdullah, 2019)

This overexpression of EGFR occurred during the first period of experiment at 10 days, then decreased at 20 days; this decreasing was related to the proliferative role in complete tissue healing at 20 day, and this was consistent with study of Dharmani *et al*. (2013) who found that the mixture of eight probiotic bacteria including Lactobacilli, Bifidobacteria, and Streptococcus species heals acetic acid-induced gastric ulcer in rats and the gene expression of EGF and VEGF in 7 days more than in 14 day. This study was also compatible with the study of Zhang *et al*. (2012) who studied levels of EGF by immunohistochemistry in treated acetic acid gastric ulcer in rats at a different periods (1,2,4,6,10,14 and 23 days) and found a little increase on day 1 reaching a peak in 10 days. On days 14 and 23, the expression level and EGF peptide showed a little decrease.

5. Conclusion

Alcoholic extract of *P. ostrateus* has a significant effect on EGFR expression in ulcerated stomach and duodenum. Many studies on side effects, effective doses, mode of preparation on *P. ostreatus* should be done to improve it as an alternative treatment for peptic ulcers.

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Total Phenolic Content, Antioxidant and Antimicrobial Activity of *Ruta chalepensis* L. Leaf Extract in Al-Baha Area, Saudi Arabia

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Abstract

This study was conducted to evaluate the phenolic content, antioxidant activity and antimicrobial activity of *Ruta chalepensis* L. leaf extract grown in Al-Baha area, Saudi Arabia. The collected fresh leaves were air-dried, powdered, and extracted with ethanol, petroleum ether, chloroform, ethyl acetate and n-butanol. The total phenolic content, antioxidant activity and antimicrobial activity against *Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus, Bacillus cereus* and *Candida albicans* were determined. The results indicated the maximum total phenolic content in n-butanol extract (2580.47 mg/gm) and the minimum in chloroform extract (18.93 mg/gm), while the antioxidant activity ranged between 34.83% in ethanol extract and 88.13% in n-butanol extract. The ethanolic extract showed antibacterial activity against *P. aeruginosa* and *S. aureus* (10 mm inhibition zone), while *B. cereus* was resistant (5 mm inhibition zone), and the petroleum ether, chloroform and ethyl acetate extracts showed no antibacterial activity, whereas n-butanol extract was active against *B. cereus* (10 mm inhibition zone). All extracts except ethanol showed antifungal activity against *Candida albicans* (10 mm inhibition zone). There is a large number of phenolic compounds that exhibit antibacterial effect, which are synthesized by various plants including many medicinal plant species employed in traditional medicine. The biological activity of the plant depends on factors such as plant part, geographical source, soil conditions, harvest time, moisture and post-harvest methods. The study concluded that the leaf extract of *Ruta chalepensis* has high total phenolic content and antioxidant activity in addition to moderate antimicrobial activity, therefore this plant can be useful as a medicinal plant.

Keywords: *Ruta chalepensis*, leaf extract, phenolic content, antioxidant activity, antimicrobial activity

1. Introduction

Herbs attracted the industries of biotechnology, cosmetics, pharmaceutical, and food as an ancient source of medicine, flavouring, beverages, dyeing, fragrances, and cosmetics (Zaidi and Dahiya, 2015). The use of plants and plant products as medicinal products could be traced back to the beginning of human civilization (Bharathi *et al*., 2014). Natural substances with antimicrobial and antioxidant properties from essential oils and plant extracts supplied as food components or specialized pharmaceuticals to human are increasingly of interest (Shafique *et al.*, 2011). Most natural plants containing a wide variety of phytochemical constituents are a major source of antioxidants that affect the decrease in the possible stress caused by reactive oxygen species. Natural antioxidants may have free-radical scavengers, reduction agents, possible pro-oxidant metal complexes, and single-single-oxygen quenches (Basoudan *et al.*, 2019). Medicinal plants and plant-based products that contain a wide variety of free radical scavenging molecules such as phenolic compounds, nitrogen compounds, vitamins, terpenoids, and some other endogenous metabolites are rich in antioxidant activity (Zheng and Wang, 2001; Cai *et al.*, 2003). The frequent usage of the common antibiotics and therapeutic compounds for the prevention of disease-causing pathogens lead to the emergence of microbial resistance to antibiotics, which caused side effects such as immune suppression, allergic reactions and hypersensitivity reactions respectively, therefore, there is an urgent need for the invention of novel molecules with fewer side effects (Almalki, 2017).

Ruta chalepensis, as a member of the family *Rutaceae*, is used for treating a variety of diseases in traditional medicine in many countries (Pollio *et al.*, 2008). It is an important medicinal plant well recognized in the Mediterranean area as well as in some temperate and tropical countries, and well known for its strong biological activities (Kacem *et al*., 2015). This perennial herb was found to possess potent antimicrobial activities against Gram-positive and Gram-negative bacteria, in conjunction with the presence of phenolic compounds (Kacem *et al.*, 2015; Amdouni *et al.*, 2016). The leaves and young stems of *R. chalepensis* have been reported to contain alkaloids, flavonoids, phenols, amino acids, furanocoumarins, and

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saponins (Cowan, 1999; Alemayehu *et al.*, 2019). The biological activities of *R. chalepensis* are frequently utilized in herbal therapy and the plant is used as a promoter of menstruation, treatment of hypertension, a topical treatment for ear aches and headaches, and an as external treatment in the form of a skin antiseptic and insect repellent (Guarrera, 1999; Steenkamp, 2003), and these plants are a source of huge variety of natural products with antibacterial, antifungal, antioxidant, spasmolytic, antihelmintic, emmenagogue, antitumoral, analgesic, anti-inflammatory, and antidepressant activities (Raghav *et al*., 2006). Rue's active ingredients have antifungal and insecticidal properties that could prove beneficial to agriculture as well (Emam et al., 2010). In Saudi Arabia, a decoction of its aerial parts is used as an analgesic and antipyretic and for the treatment of rheumatism and mental disorders (Iauk *et al*., 2004).

The Kingdom of Saudi Arabia is gifted with a wide range of flora consisting of a large number of medicinal herbs, shrubs, and trees (El-shabasy, 2016). The study was conducted to evaluate the phenolic content and antioxidant activity of different extracts of the leaves of *R. chalepensis* and to ensure its pharmaceutical value as it was used as a medicinal plant traditionally.

2. Materials and Methods

2.1. Sample Collection and Preparation

Fresh leaves of *R. chalepensis* were collected from Al-Baha area, Kingdom of Saudi Arabia. The leaves were washed with fresh water to remove the soil and dust particles, and subjected to air-drying under shade for three weeks until they were completely dried, then ground into fine particles using an electric grinder.

2.2. Preparation of Ethanolic Extract of Samples

Two hundred grams (200 gm) of powdered sample were weighed into a clean dried flask or beaker (2-3 L size). Two-thousand milliliters (2000 ml) of 80% ethanol (800 ml ethanol+200 ml distilled water) were added. The mixture was well mixed and soaked for 2 days at room temperature. The mixture was then filtered with filter paper (Whatman no. 4). This procedure was repeated three times to ensure that all contents were extracted with ethanol. The filtrates were collected and allowed to air dry for 10 days, and the extract was stored in a coloured bottle at 4-6°C till analysis.

2.3. Fractionation of the ethanolic extract by liquid–liquid extraction

The ethyl alcohol extract was soaked in 200 mL distilled water and extracted consecutively using different solvents (petroleum ether, chloroform, ethyl acetate, and *n*-butanol, respectively) for 7 days at room temperature to produce extracts. The solvents are chosen depending on their polarity difference. The filtrate was dried by using a rotary evaporator at room temperature, and stored at 4-6°C till used.

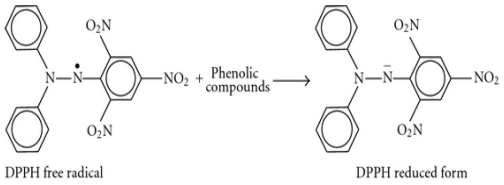
2.4. Total Phenolic Content (TPC)

The concentration of phenolics in the leaves extracts was determined using a spectrophotometric method (Singleton *et al*., 1999), with some modifications. Sample solutions of the ethanolic extracts in the concentration of 1 mg/ml were used in the analysis. The reaction mixture was prepared by mixing 0.5 ml of sample solutions of fractions, 2.5 ml of 10% Folin-Ciocalteu's reagent dissolved in water and 2.5 ml of 7.5% $NaHCO_3$. Blank was concomitantly prepared, containing 0.5 ml methanol, 2.5 ml 10% Folin-Ciocalteu's reagent dissolved in water and 2.5 ml of 7.5% of $NaHCO_3$. The samples were thereafter incubated at 30°C for 90 min. The absorbance was determined using a spectrophotometer at λmax = 765 nm. The samples were prepared in triplicate for each analysis. The same procedure was repeated for the standard solution of gallic acid and the calibration line was constructed. Based on the measured absorbance, the concentration of phenolics was read (mg/ml) from the calibration line; then the content of phenolics in extracts was expressed in terms of gallic acid equivalent (mg of GAE/gm of extract).

2.5. Total Antioxidant Activity

2.5.1. Free Radical Scavenging Assay

The antioxidant assay used was based on the scavenging ability of the antioxidant (s) in plant extracts towards the stable free radical 1,1-diphenyl-2-picrylhydrazile (DPPH), which is deep purple, to form the corresponding hydrazine with the accompanying change of colour to light purple or golden yellow.



2.5.2. Free radical Scavenging Procedure

This method was carried out according to Shyur *et al.* (2005) with some modifications. Stock solution was prepared by dissolving 1mg of the sample in 1ml of absolute ethanol (98%). The stock solution was diluted to final concentrations of 100, 50, 25, 12.5, 6.25, 3.125, 1.5625 µg/ml in ethanol. 0.9ml of Tris-HCl and 1ml of 0.1 mM DPPH in methanol solution were added to each concentration and incubated at room temperature in the dark for 30 minutes. The absorbance of the resulting mixture was measured at 517 nm and converted to percentage antioxidant activity using the formula below:-

Scavenging activity (DPPH scavenged) (%) = $\frac{(Ac - As)}{Ac} \times 100$

Where: Ac= Absorbance of control; As = Absorbance in the presence of the sample of extract

A solution of 0.9 ml Tris-HCl+ 0.1ml absolute ethanol+ 1ml absolute ethanol was used as a blank, while solution of 0.9 ml tris-HCl+0.1ml absolute ethanol+1ml DPPH was used as a control. Freshly prepared DPPH solution exhibits a deep purple colour with a maximum absorbance at 517 nm. The purple colour disappears when an antioxidant is present in the medium. Thus, the change in the absorbance of the reduced DPPH was used to evaluate the ability of compound to act as a free radical scavenger.

2.6. Antimicrobial activity test

2.6.1. Microbial organisms

Bacterial strains of *Bacillus subtilis* (NCTC 8236), *Staphylococcus aureus* (ATCC 25923), *Escherichia coli* (ATCC 25922) and *Pseudomonas aeruginosa* (ATCC 27853), in addition to a fungal strain *Candida albicans* (ATCC 7596) were used in this study for antimicrobial activity test.

2.6.2. Preparation of bacterial suspensions

Aliquots (1 ml) of a 24 hr broth culture of the bacteria were aseptically distributed onto nutrient agar slopes and incubated at 37ºC for 24 h. The bacterial growth was harvested and washed off with 100 ml sterile normal saline to produce a suspension containing about 10^8- 10^9cfu/ ml, which was stored at 4°C till used. The average number of viable organisms/ml of the stock suspension was determined employing the surface viable counting technique (Miles and Misra, 1938). Serial dilutions of the stock suspension were made in a sterile normal saline solution and 0.02 ml volumes of the appropriate dilution were transferred by micropipette onto the surface of dried nutrient agar plates, which were allowed to stand for 2 h at room temperature for the drops to dry and then incubated at 37°C for 24 h. After incubation, the number of developed colonies in each drop was counted. The average number of colonies per drop (0.02 ml) was multiplied by 50 and the dilution factor to get the viable count of the stock suspension, expressed as colony forming units (cfu)/ml suspension. Each time a fresh stock suspension was prepared. All the above experimental conditions were maintained constant so that suspensions with very close viable counts would be obtained.

2.6.3. Preparation of fungal suspension

The fungal cultures were maintained on Sabouraud dextrose agar, incubated at 25°C for 4 days. The fungal growth was harvested and washed with sterile normal saline and finally suspended in 100 ml sterile normal saline and stored at 4°C until used.

2.6.4. Agar disc diffusion

The disc diffusion method was used to screen the antibacterial activity of plant extracts and performed by using Mueller Hinton agar (MHA) and Sabouraud dextrose agar (SDA). The experiment was carried out according to Boudjema *et al.* (2018). Bacterial and fungal suspensions were diluted with a sterile physiological solution to 10^8cfu/ ml (turbidity = McFarland standard 0.5). One hundred microliters of bacterial and fungal suspensions were swabbed uniformly on the surface of MHA and SDA and the inoculum was allowed to dry for 5 min. Sterilized filter paper discs (Whatman No.1, 6 mm in diameter) were placed on the surface of MHA and SDA and soaked with 20 µl of a solution of each plant extract. The inoculated plates were incubated at 37°C for 24 h in an inverted position. After incubation, the antimicrobial activity was determined by measuring the diameter of the inhibition zone surrounding each disc. The antimicrobial activity results were expressed in terms of the diameter of the inhibition zone as follows: <9 mm zone (resistant strain); 9-12 mm (partially sensitive strain);13-18 mm (sensitive strain); >18 mm (very sensitive strain).

2.7. 2.7. Statistical Analysis

The statistical analyses were performed using Statistical Analysis Systems (SAS, Ver. 9, SAS Institute Inc., Cary, NC, USA) and the results were presented as the mean ± standard deviation (SD) of three replicates. All the data were statistically assessed using the General Linear Model (GLM) and the significant difference was performed using Duncan multiple range test at $P \leq 0.05$.

3. Results and Discussion

3.1. Total phenolic content (TPC)

From results in Table 1, TPC of *R. chalepensis* leaf extract is significantly ($P<0.001$) higher in n-butanol extract (2580.47mg/gm) followed by ethanol (944.63 mg/gm), petroleum ether (720.93 mg/gm), ethyl acetate (717.13 mg/gm) and lower in chloroform extract (18.93 mg/gm). Total phenolic content is affected by the polarity of the solvent used for extraction which is being maximum in polar solvents and minimum in non-polar solvents, therefore TPC of *R. chalepensis* is polar and extracted well with polar solvents. Ereifej *et al*. (2015) extracted TPC using methanol at 60°C and reported that the results depict that all plants contain appreciable amounts of phenolic compounds and that TPC of *R. chalepensis* leaves/flowers extracted with methanol was 1328.8 mg GAE/100gm extract. Amdouni *et al*. (2016) reported that the pattern and degree of total phenolic concentration (TPC) differed with the organ type and treatment. In all circumstances, TPC was much higher in leaves and stems than in roots. These results are in agreement with the findings of Defa *et al.* (2017) who reported that TPC in *R. chalepensis* is polar and highly extracted by the polar solvents (like dissolves like). Ouerghemmi *et al*. (2017) informed that the total phenolic content was more organ- than provenance-dependent and varied 0.2-168.91 mg of GAE/gm dry weight. Pavi´c *et al*. (2019) established that the highest TPC (38.0±0.11 mg GAE/gm dry matter) was recorded in the rue extract obtained at 30°C, 20% water for 90 min, whereas the lowest content (28.84±1.96 mg GAE/gm dry matter) was found in rue extract obtained at 30°C, 20% water for 30 minutes.

Table1. The percentage of total phenolic content of *Ruta chalepensis* leaf extracted by different solvents

Extract	Total phenolic content (mg/gm)
Ethanol	944.63 ± 71.42^{c}
Petroleum ether	720.93 ± 88.31^{b}
Chloroform	18.93 ± 10.84^{d}
Ethyl acetate	717.13 ± 258.57^{b}
n-butanol	2580.47 ± 175.59^{a}

The values are presented as the mean ± the standard deviation (n = 3). Mean values with different letters within a column are significantly different ($p<0.001$).

3.2. Total antioxidant activity

The degree of discolouration of the deep purple colour of DPPH radical, as it is reduced, indicates the radical scavenging potential of the antioxidant (Singh *et al*., 2002). The DPPH radical scavenging effects of extracts of *R. chalepensis* in different solvent systems are shown in Table 2. The scavenging effect ranged between 34.83±11.98% in ethanol extract and 88.13±2.46% in n-

butanol extract (P<0.001), with the scavenging effect decreasing in the order of n-butanol>ethyl acetate>petroleum ether>chloroform>ethanol. Amdouni *et al*. (2016) outlined that the total antioxidant capacity of *R. chalepensis* was higher in leaves (13 mg GAE g-1 DW) than in stems (11 mg GAE g-1 DW) and roots (10 mg GAE / gm DW) and reached its maximum under salt stress (24.26 mg GAE / gm DW) in leaves of plants treated with 100 mM NaCl, followed by stems (17 mg GAE / gm DW) and roots (16 mg GAE/gm DW). The IC_{50} values showed that the ethyl acetate IC_{50} was 0.00165 µg/ml, whereas the n-butanol IC_{50} was 0.000299 µg/ml, while for the rest of solvents the test was not carried out, suggesting that the higher the antioxidant activity value, the lower the IC_{50} value (Table 2). Such findings are lower than those reported by Amdouni *et al*. (2016) for *R. chalepensis* leaf extract (IC_{50} = 0.097 µg/mL) and Ben Sghaier *et al*. (2018) for which IC_{50} was 54.1±1.5 µg/ml for methanol extract compared to aqueous extract (IC_{50}=73.6±2.6 µg/ml). Ouerghemmi *et al.* (2017) recorded 189.61 mg GAE/gm DW and 172.63 61 mg GAE/gm DW for spontaneous and cultivated *Ruta* leaves, respectively, and 169.6 mg GAE / gm DW and 161.36 mg GAE / gm DW for spontaneous and cultivated *Ruta* flowers, respectively. Alotaibi *et al*. (2018) recorded the best antioxidant activity of *R. chalepensis* to be obtained by D2 (ethyl acetate and n-butanol) extract (94.28%, IC_{50}=56.6 mg/ml), while ethanol and D1 (ether and chloroform) extracts displayed an antioxidant activity of 87.51%, IC_{50}=320.7 mg/ml, and 80.37%, IC_{50}=414.9 mg/ml, respectively, and all extracts had very good antioxidant activity. Similar results of 68.23±0.89 were reported by Basoudan *et al*. (2019). These results are consistent with the findings of Pavi´c *et al*. (2019) who reported that Rue extract obtained at 30°C, 20% water for 90 min, exhibited the highest antioxidant activity (72.53±0.31%) among the extracts studied, while the lowest antioxidant activity (57.54±0.15%) was in rutin extracted at 70°C, 27% water for 52 min; and in disagreement with Alemayehu *et al*. (2019) who reported that at the concentration of 1000 µg/ml used, the scavenging effect of *R. chalepensis* extracts on the DPPH radical decreased in the order of methanol>acetone>ethyl acetate>n-hexane (93.851±0.148%>78.937±0.961% > 76.590±0.131% > 64.499±0.679, respectively).

Table 2. DPPH radical scavenging activity (%) of leaves extracts of *Ruta chalepensis* leaf extracted with different solvents

Extract	Radical scavenging concentration (%)	IC_{50}
Ethanol	34.83±11.98^{c}	ND
Petroleum ether	43.53±2.64b^{c}	ND
Chloroform	43.10±1.51b^{c}	ND
Ethyl acetate	52.17±4.54^{b}	0.00165
n-butanol	88.13±2.46^{a}	0.000299

The values are presented as the mean ± the standard deviation (n = 3). Mean values with different letters within a column are significantly different (p<0.001).

ND = Not determined

3.3. Antimicrobial activity

Medicinal plants have become part of alternative medicines worldwide because of their potential health benefits and can be consumed or directly used to treat infections (Gloria-Garza *et al*., 2013). The biological activity of the plant depends on many factors, such as plant part, geographical source, soil conditions, harvest time, moisture and post-harvest methods (Gloria-Garza *et al*., 2013). High temperatures during tissue grinding may denature certain chemical constituents. For the maximum recovery of bioactive compounds, different concentrations of solvents or different solvents alone or combinations are used, because different plants constitute different compositions of active compounds (Amabye and Shalkh, 2015). The leaves of *R. chalepensis* extracted with different solvents were tested for antimicrobial activity using agar disc diffusion method. The findings reported in Table 3 showed that the ethanolic extract demonstrated antibacterial activity against *P. aeruginosa* and *S. aureus* with inhibition zones of 10 mm, while *B. cereus* was resistant (5 mm inhibition zone). Petroleum ether, chloroform, and ethyl acetate extracts displayed no antibacterial activity against all of the bacteria studied, and only n-butanol extract was active against *B. cereus* (inhibition zone of 10 mm). Our results are consistent with the findings of Merghache *et al*. (2008) who reported the essential oil in the aerial part of *R. chalepensis* to be unsuccessful in the inactivation of *E. coli*, *P. aeruginosa*, *S. aureus* and *L. monocytogens*; and in disagreement with the findings of Alemayehu *et al*. (2019) who reported that methanol, acetone, hexane, and ethyl acetate extracts possess antimicrobial activity against *B. cereus*, *S. aureus*, *S. typhi* and *E. coli* with ≥ 11 mm mean zone of inhibition, and not in line with Ouerghemmi *et al*. (2017) who found that wild and cultivated *Ruta* organ extracts exhibited marked antibacterial activity, with spontaneous *Ruta* stem extracts being effective against *S. aureus* and *P. aeruginosa* (16.3 mm and 17.7 mm inhibition zone, respectively) and the cultivated *Ruta* stem extracts showed the highest antibacterial activity against *E. coli*. The methanol extract of *R. chalepensis* leaf at 5 mg/disk displayed a potent inhibiting activity against *C. perfringens* and *E. coli*, and no or poor activity against *Bifidobacterium bifidum*, *Bifidobacterium longum*, *L. acidophilus*, and *L. casei*, while the chloroform extract exhibited a potent activity against *C. perfringens* and *E. coli*, whereas butanol and water extracts showed no activity (Jang-Hee *et al*., 2005). *R. chalepensis* methanol extract showed MICs of 250 µg/ml and 3.9 µg/ml, and induced a maximum of 63% and 94% growth inhibition against *S. mutans*, as measured by the MTT reduction and CFU methods, respectively, whereas the vehicle control and medium alone (both free from plant extract) did not alter bacterial growth (Gloria-Garza *et al*., 2013). Babu Kasimala *et al*. (2014) found that the acetone extract of *R. chalepensis* showed maximum inhibition (8.5 mm) against Gram negative bacteria, while ethanol extract showed maximum inhibition against Gram positive bacteria (8 mm). The inhibitory effect of plant extracts against bacterial pathogens was due to their phenolic content and the inhibitory effect of phenolic compounds could be explained by cell membrane adsorption, enzyme interaction, substrate and metal ion deprivation (Ouerghemmi *et al*., 2017). Alotaibi *et al*. (2018) reported that except for *Proteus vulgaris* and *P. aeruginosa*, the obtained results revealed that all *R. chalepensis* solvent extracts (ethanol and collected successive extracts of ether and chloroform, ethyl acetate and n-butanol, respectively) possessed antibacterial activity against all Gram-negative

and Gram-positive bacteria under investigation, with the activity being variable according to the solvent. Tedila *et al*. (2019) reported that among the solvents used to extract the biologically active substances from *R. chalepensis*, ethanol and methanol were the best solvents; followed by acetone and the least were diethyl ether and hexane, and they concluded that the extraction of medicinal plants with different solvents could produce different results depending on the ability of the solvent. Al-Majmaie *et al*. (2019) stated that rutin (1) and its two derivatives (2 and 3) from *R. chalepensis* flowers showed varying degrees of activity against all tested organisms in the resazurin microtitre assay with compound 3 being the most potent, and the order of antimicrobial potency among these compounds was 3>2>1. All extracts except ethanol significantly (P<0.001) showed antifungal activity against *Candida albicans* with a 10 mm inhibition zone (Table 3). The essential oil of the aerial portions of *R. chalepensis* demonstrated antifungal activities against *Aspergillus niger*, *Aspergillus flavus*, *Alternaria sp*., *Trichoderma sp*. and *Candida albicans* (Merghache *et al*., 2008). Such results are consistent with those of Emam *et al*. (2010) who reported that the ethanolic extract (80%) of *R. chalepensis* leaves exhibited antifungal activity against the three phytopathogenic fungi *Rhizoctonia solani*, *Sclerotium rolfsii* and *Fusarium solani*, which cause root-rot and wilt diseases in a number of economically important food crops such as potato, sugar beet, and tomato, when examined with the disk diffusion technique, with inhibition zone diameters being 10, 18, and 12 mm, respectively. Sepahvand *et al*. (2018) reviewed the most common dermatophytosis medicinal plants in traditional medicine and concluded that the medicinal plants including *R. chalepensis* are the most effective plants on dermatophytes that have been identified to date.

Table 3. Antimicrobial activity (inhibition zone in mm) of *Ruta chalepensis* leaf extract determined by agar disc diffusion assay (100 mg/ml)

Microorganisms	Extract					S.L.
	Ethanol	Petroleum ether	Chloroform	Ethyl acetate	n-butanol	
Gram-positive						
E. coli	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	-
P. aeruginosa	10.00±0.00^{a}	0.00±0.00^{b}	0.00±0.00^{b}	0.00±0.00^{b}	0.00±0.00^{b}	***
Gram-negative						
S. aureus	10.00±0.00^{a}	0.00±0.00^{b}	0.00±0.00^{b}	0.00±0.00^{b}	0.00±0.00^{b}	***
B. cereus	5.00±0.07^{ab}	0.00±0.00^{b}	0.00±0.00^{b}	0.00±0.00^{b}	10.00±0.00^{a}	N.S.
Fungus						
C. albicans	0.00±0.00^{b}	10.00±0.00^{a}	10.00±0.00^{a}	10.00±0.00^{a}	10.00±0.00^{a}	***

The data are presented as mean ±standard deviation (n=3) Means in each row bearing similar superscripts are not significantly different (P>0.05) ,*** = P<0.001

N.S. = Not significant, S.L. = Significance level

4. Conclusion

In this study, *R. chalepensis* leaf extracted with ethanol, petroleum ether, chloroform, ethyl acetate, and n-butanol were evaluated for total phenolic content in addition to antioxidant and antimicrobial activity evaluation. The results indicated that the overall phenolic content decreased by n-butanol extract (2580.47mg/gm) > ethanol (944.63 mg/gm)>petroleum ether (720.93 mg/gm) > ethyl acetate (717.13 mg/gm) > chloroform extract (18.93 mg/gm). The plant has a high antioxidant activity and mild antimicrobial activity, although the essential oil had antimicrobial activity against the organism in the study in a previous study of the same authors (unpublished data). Since the area of Al-Baha is in rich *R. chalepensis*, the plant could be a potential source of natural bioactive molecules that could substitute for synthetic antioxidants and function as a source of antibacterial agents in the food industry and can serve as a medicinal plant

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Curative Potential of Nigerian Medicinal Plants in COVID-19 Treatment: A Mechanistic Approach

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Abstract

COVID-19 is a highly infectious and severe acute respiratory disorder caused by a pathogenic virus known as severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). Its clinical manifestations include fever, fatigue, cough, shortness of breath, and other complications. Mode of actions of SARS-CoV-2 includes hyper-inflammation characterized by a severe and fatal hyper-cytokinaemia with multi-organ failure; immunosuppression; reduction of angiotensin-converting enzyme 2 (ACE2) to enhance pulmonary vascular permeability and damage the alveoli and activated by open reading frame (ORF)3a, ORF3b, and ORF7a via c-Jun N- terminal kinase (JNK) pathway which induces lung damage. These mechanisms of action of the virus can be mitigated by combine therapy of the medicinal herbs based on their pharmacological activities. At present, there is no effective treatment or vaccine that can mitigate/inhibit coronavirus. Available clinical intervention for COVID-19 is only palliative and limited to support. Thus, there is an exigent need for effective and non-invasive treatment. The article critically assesses the proposed mechanism of actions of SARS-CoV-2 and presents Nigeria based medicinal plants which have pharmacological and biological activities that can mitigate the hallmarks of the pathogenesis of COVID-19. Since the clinical manifestations of COVID-19 are multifactorial and co-morbidities, we strongly recommend the use of combined therapy with two or more herbs with specific therapeutic actions being administered to combat the mediators of the disease.

Keywords: COVID-19; SARS-CoV-2; Phytochemicals; mechanistic approach; medicinal plants

1. Introduction

Novel Coronavirus disease 2019 (COVID-19) is a highly infectious and severe acute respiratory disorder caused by an infectious virus called SARS-CoV-2 which is transmitted to humans via contact and/or feeding on infected animals. The COVID-19 clinical manifestations are very similar to viral pneumonia such as fever, fatigue, cough, shortness of breath, and other complications. According to reports obtained on WHO and NCDC websites as of 16th May 2020, the Coronavirus breakout in Wuhan, a city in Hubei Province of China in November 2019 has spread to many countries in the world. This global pandemic has forced many nations to lock down their social activities, which in turn has adverse effects on the economy. Globally, more than 3,500,000 people have been confirmed infected with over 250,000 deaths. Nigeria is one of the countries seriously affected by the virus having over 6000 cases and more than 190 mortalities (WHO, 2020; NCDC, 2020). Thus, there is an exigent need for effective and non-invasive treatment.

Coronaviruses (SARS-CoV) are non-segmented positive-sense single-stranded RNA viruses with a large viral RNA genome of diameter 80–120 nm (figure1). They belong to the family of Coronaviridae, in the subfamily Orthocoronaviridae which consists of four genera namely: Alpha, Beta, Gamma, and Delta coronavirus (Chan *et al.*, 2013). Some of the proposed modes of actions of SARS-CoV-2 include hyper-inflammation characterized by a sudden and fatal hyper-cytokinaemia with multi-organ failure (Huang *et al.*, 2020); immunosuppression; reduction of Angiotensin-Converting Enzyme 2 (ACE2) to enhance pulmonary vascular permeability and damage the alveoli (Li and Clercq, 2020) and activated by ORF3a, ORF3b, and ORF7a via JNK pathway which induces lung damage (Liu *et al.*, 2014).

At present, there is no effective treatment or vaccine that can mitigate/inhibit coronavirus. Available clinical interventions for COVID-19 are only palliative and limited to support. Many research groups around the world are currently focusing on developing novel treatments such as vaccines and antivirals. This article critically assesses the proposed mechanism of actions of SARS-CoV-2 and presents Nigeria based medicinal plants which have

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pharmacological and biological activities that can mitigate the hallmarks of the pathogenesis of COVID-19. Since the clinical manifestations of COVID-19 are multifactorial and co-morbidity, we strongly recommend the use of combined therapy such that two or more herbs with specific therapeutic actions are administered. This could provide a desired medical intervention against the mediators of the disease.

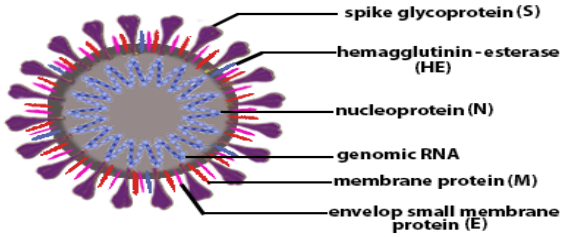


Figure 1. Structure of SARS-CoV-2

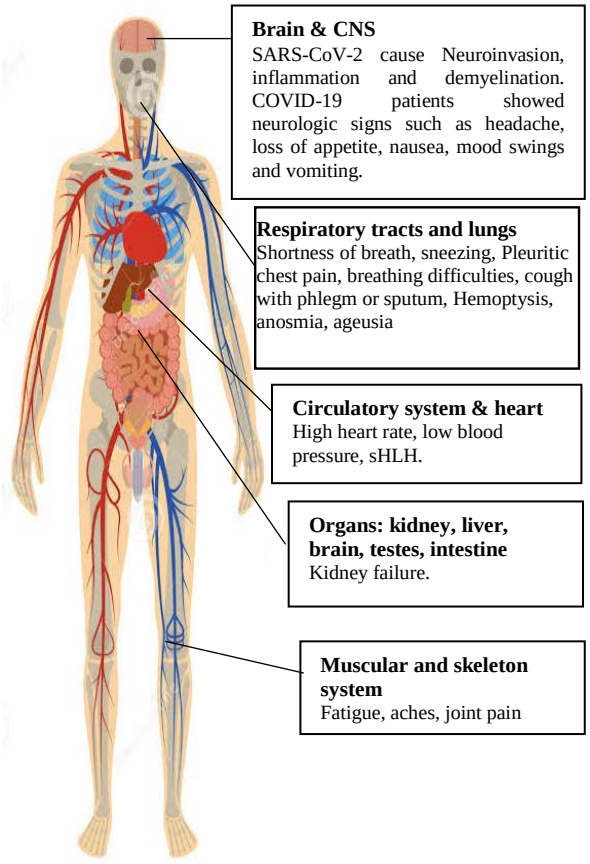
2. History and Prevalence

COVID-19, novel coronavirus pneumonia is ranked at the ninth deadliest world pandemic ever experienced in the world was first recorded in late 2019 at a Chinese city called Wuhan. Since its first outbreak, the disease has spread to every continent of the world affecting many nations. This highly infectious and severe acute respiratory disease is transmitted to humans and animals by a pathogenic virus called SARS-CoV-2. Reports from epidemiological findings documented that the disease is comparatively less life-threatening and not common in children (Lu *et al.*, 2020; Wu and McGoogan 2020). Updates by WHO on 16th May 2020 showed that COVID-19 poses a major threat to global public health. The data revealed that more than 3,500,000 confirmed cases of SARS-CoV-2 infection and over 250,000 deaths globally since the first case were reported in late 2019. In Nigeria, a country located in the western region of Africa, over 6000 cases had been confirmed with 190 mortalities (WHO, 2020; NCDC, 2020).

COVID-19 has spread rapidly from Wuhan in China to all the continents of the world within four weeks. This confirms that COVID-19 has a very high prevalence, and the global population is generally susceptible to SARS-CoV-2. Using the IDEAL model, Majumder et al. documented that the basic reproduction number (R0) of SARS-CoV-2 is 2.0–3.3 (Majumder and Kenneth, 2020), while Wu *et al.* (2020) reported that the R0 is between 2.47 and 2.86 using the SEIR model (Majumder and Kenneth, 2020). R0 is a parameter for measuring the transmission potential of contagious diseases. It indicates the average number of secondary infections that may occur in an entirely susceptible population (Remais, 2010). The values of R0 may vary between research groups due to many factors such as duration of infectiousness, probability of infection being transmitted during contact, and rate of contacts in the host population. The calculated R0 values of other beta coronaviruses are 2.2–3.6 (Lipsitch *et al.*, 2003). This revealed that SARS-CoV-2 has relatively high communicability. The median age of cases reported in China was 47 years, 3% of the cases were aged people (≥80 years), 87% of the cases were people between the age of 30 and 79 years. Forty-two percent of the cases were female, suggesting that males may be more susceptible to SARS-CoV-2 (Guan *et al.*, 2020; Wu and McGoogan 2020).

3. Pathogenesis of Coronavirus disease 2019 (COVID-19)

COVID-19, a severe acute respiratory viral infection in humans caused by SARS-CoV-2 has an average incubation period of 3 days (Guan *et al.*, 2020). The most common clinical features of COVID-19 are very similar to other viral pneumonia which include fever, fatigue, cough, shortness of breath, and other complications; organ failure and death were recorded in severe and critical cases (Figure 2) (WHO, 2020). These symptoms are markedly expressed in adults probably due to chronic underlying diseases such as heart diseases, neurodegenerative disorders, diabetes, or hypertension (Chen *et al.*, 2020). Transmission of the virus among humans occurs when there is a penetration of infected aerosols from respiratory droplets, cough, or sneeze into the lungs via inhalation through the nose or mouth.



COVID-19 has been reported to have a higher mortality rate of about 3.7% when compared with influenza with >1% mortality rate (WHO, 2020). Some scientific evidence showed that some sets of severe COVID-19 cases might have a cytokine storm syndrome and respiratory failure due to acute respiratory distress syndrome (ARDS) which is the major cause of death (Ruan *et al.*, 2020). Viral infections are the major factor that initiates secondary haemophagocytic lymphohistiocytosis (sHLH) (Ramos-casals *et al.*, 2014). sHLH also known as Macrophage Activation Syndrome (MAS) is a life-threatening medical condition which comprises a heterogeneous group of

hyper-inflammatory syndrome occurring when there is an infraction in the interplay of genetic predisposition and activators such as infections. It is characterized by a sudden and severe hyper-cytokinaemia due to inappropriate survival of histiocytes and cytotoxic T-lymphocytes and ultimately leads to haemophagocytosis, multi-organ failure, and high mortality (Henter *et al.*, 2002). Fundamental characteristics of sHLH are cytopenias, persistent fever, and hyper-ferritinaemia; pulmonary involvement occurs in approximately 50% of patients (Seguin *et al.*, 2016).

However, the immunosuppression pathway depicting how SARS-CoV-2 affects the immune system has not been fully elucidated. Nevertheless, MERS and SARS have been reported to evade immune detection and weaken immune responses. During viral infection, host factors produce an immune response against viruses. CD4+ and CD8+ are important T cells which perform a pivotal role in mitigating the virus and decreasing the chance of acquiring autoimmunity/inflammation (Cecere *et al.*, 2012). The CD4 + T cells enhance the synthesis of viral-specific antibodies by activating T cell-dependent B cells, while CD8+T cells are cytotoxic and wipe out virus-infected cells. Approximately, 80% of total inflammatory cells in the pulmonary interstitial in SARS-CoV infected patients are CD8+T cells. They perform important functions in scavenging and coronaviruses in infected cells (Maloir *et al.*, 2018). Furthermore, T helper cells produce proinflammatory cytokines through the NF-kB signaling pathway (Manni *et al.*, 2014).

4. Modes of action of SARS-CoV-2

Using sequencing technology, the analysis of SARS-CoV-2 genetic sequences showed that the complete genome sequence recognition rates of SARS-CoV and bat SARS coronavirus (SARSr-CoV-RaTG13) were 79.5% and 96.2%, respectively (Chen *et al.*, 2020). Like other coronaviruses, SARS-CoV-2 has specific genes in ORF1 regions that stimulate proteins for viral replication, spikes formation, and nucleocapsid (van Boheemen *et al.*, 2012). The SARS-CoV-2 enter into and affect the host cell by undergoing a few steps of modifications similar to other kinds of beta-coronaviruses. Thereafter, it binds to the ACE2 receptor in the alveoli of the lungs and respiratory epithelium (Liu *et al.*, 2020a, b). Binding of SARS-CoV to the receptor results in the mobilization of cellular proteases to cleave the S protein into S1 and S2 domains. These cellular proteases include cathepsins, human airway trypsin-like protease (HAT), and transmembrane protease serine 2(TMPRSS2) that split the spike protein and establish further penetration changes (Glowacka *et al.*, 2011; Bertram *et al.*, 2020). This cleavage enhances the activation of S2 via a conformational change thus allows the interpolation of the internal fusion protein (FP) into the membrane mediating the entrance of the virus into the cell.

There is a probability that SARS-CoV-2 employed a similar mechanism as SARS-CoV because its receptor-binding domain (RBD) binding motif consists of the nucleotides associated with ACE2. After SARS-CoV-2 gained entrance in its host cell, ACE2 is cleaved and ADAM metallopeptidase domain 17 (ADAM17) shed by it into the extra membrane space. This may lead to the conversion of angiotensin I to angiotensin II by ACE2, a negative regulator of the renin-angiotensin pathway, thus increasing pulmonary vascular permeability and damaging the alveoli (Chan *et al.*, 2020). After SARS-CoV-2 proteins are translated in the host cell, ORF3a protein which codes for a Ca^{2+} ion channel that is related to SARS-CoV-2 is synthesized. It interacts with TRAF3 and activates the transcription of Nuclear Factor kappa-light-chain-enhancer of activated B-cells (NF-kB) pathway, leading to the transcription of the pro-IL-1B gene (Siu *et al.*, 2019), ORF3a along with TNF receptor-associated factor 3 (TRAF3) and ORF3a mediates the inflammasome complex which contains caspase 1, Apoptosis-associated speck-like protein containing a CARD (ASC), and Nod-like receptor protein 3 (NLRP3). Second signal like the ROS production, Ca^{2+} influx, mitochondrial damage, and caspases activation converts pro-IL-1B to IL-B and results in cytokine production. Another ORF8b protein also activates the inflammasome pathway through NLRP3. This protein is longer in SARS-CoV-2 (Siu *et al.*, 2019). The E protein forming an ion channel is also involved in the overproduction of cytokines (a phenomenon known as cytokine storm syndromes which cause respiratory distress) through the NLRP3 inflammasome pathway (Nieto-Torres *et al.*, 2015).

JNK is another important pathogenic pathway of SARS-CoV. In this pathway, there is an overproduction of pro-inflammatory factors via activation of ORF3a, ORF3b, and ORF7a which may lead to increased production of proinflammatory factors, critical damage of the lung (Huang *et al.*, 2020). A cytokine profile resembling secondary haemophagocytic lymphohistiocytosis (sHLH) with a hyperinflammatory syndrome characterized by a fulminant and severe hypercytokinaemia with multiorgan failure is associated with COVID-19 disease severity. This is characterized by increased tumor necrosis factor-α, interleukin (IL)-2, IL-7, interferon-γ inducible protein 10, granulocyte-colony stimulating factor, macrophage inflammatory protein 1-α, and monocyte chemoattractant protein 1 (Huang *et al.*, 2020).

Furthermore, when compared with other kinds of respiratory syndrome coronaviruses like the Middle East respiratory syndrome coronavirus (MERS-CoV) and severe acute respiratory syndrome coronavirus (SARS-CoV), SARS-CoV-2 showed higher infectivity and transmissibility but a low mortality rate. The observed increase in virulence of SARS-CoV-2 may be due to much higher strength at which SARS-CoV-2 binds to ACE2 and mutation noted in its genome sequence. The detected changes on the SARS-CoV-2 gene include differences in orf8 and orf10 proteins, alteration on Nsp 2 and 3 proteins, shorter 3b segments, absent 8a, and longer 8b (Wu *et al.*, 2020a,b; Xu *et al.*, 2020a,b).

5. Nigerian medicinal plants with pharmacological and biological action capable of mitigating SARS-CoV-2

Various therapeutic approaches have been used since time immemorial for many health ailments, apart from the pharmacological treatment. Approximately, eighty percent of the world population still depends upon the use of herbal remedies for their health care. Nigeria and many other countries in West Africa are blessed with several varieties of medicinal plants which are of use for various purposes. This traditional method of treating ailment is

transferred from one generation to the other all over the world. Dependence on plants usage has been attributed to their affordability, effectiveness, safety, cultural preferences, and ample accessibility at all times and need. Globally, traditional healers are using various medicinal plants for the treatment of COVID-19. We, therefore, present some of the Nigeria indigenous medicinal plants with therapeutic abilities which may serve as effective treatment for COVID-19 due to their antiviral, anti-inflammatory, antioxidant, antipyretic, immunomodulatory and cytoprotective properties (figure 3).

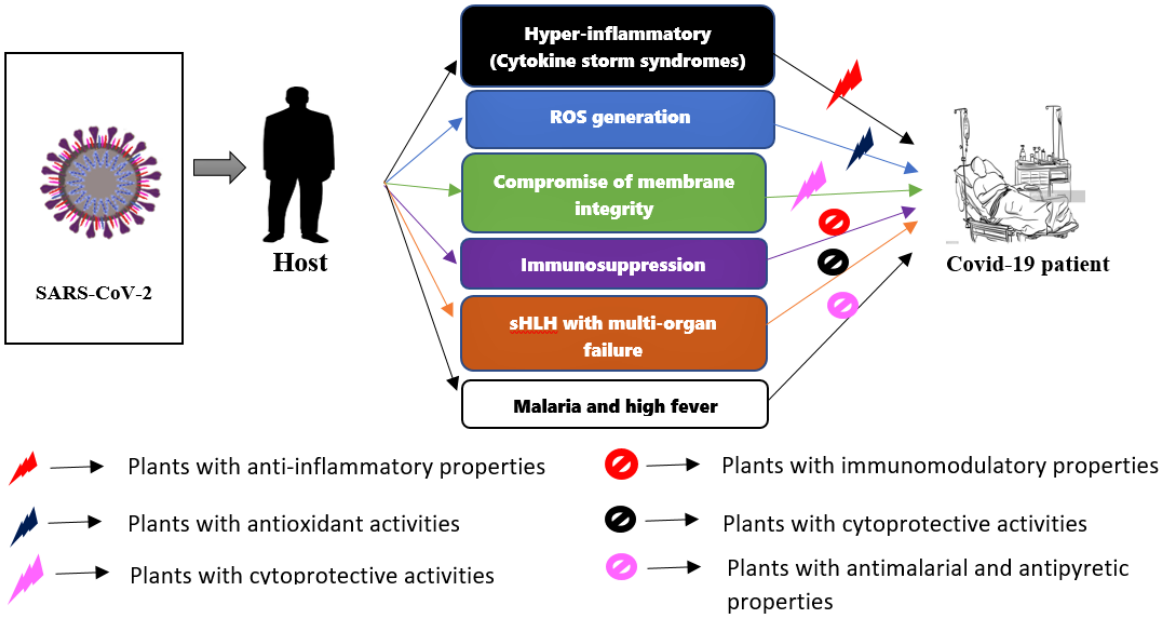


Figure 3. Pathological features of Covid-19 and possible clinical interventions by medicinal plants.

5.1. Nigerian medicinal plants with antimalarial and antipyretic properties

High fever and malaria have been reported as clinical manifestations or symptoms of COVID-19. Malaria is a global health burden caused by infection with a parasite of genus plasmodium. Scientific studies have tried to investigate the link between malaria and other diseases such as cancers, especially lymphoma, Burkitt lymphoma (caused by gamma herpes Viruses, Epstein-Barr virus), Kaposi sarcoma (caused by Kaposi sarcoma-associated herpesvirus), nasopharyngeal carcinoma and liver cancer. Nigerian indigenous medicinal plants such as *Enantiachlorantha, Khaya grandifoliola, Alstoniaboonei, Morinda lucida,* and *Azadirachta indica* are being used extensively in traditional medicine as malaria therapy. Table 1 below shows the comprehensive list of indigenous medicinal plants used for malaria therapy. The leaves, barks, roots, or whole plants are used for the treatment. Different species of the plants or parts have also been combined to formulate antimalarial herbal mixtures (Table 2). We suggest that any of these plants administered alone or in combinations may offer beneficial effects in alleviating malaria in COVID-19 patients.

Table 1. List of Nigerian medicinal plants with antimalarial properties

S/N	Botanical nomenclature	Family Names	Local Names	Common Names	Parts Used
1	*Acanthospermum hispidum*	*Compositae*	Egungun arugbo	Starrburr	Leaves, whole plant.
2	*Allium sativum*	*Liliaceae*	Ayuu	Garlic	Bulb
3	*Alstoniaboonei*	*Apocynaceae*	Ahun	Stool wood	Root, bark, leaves.
4	*Anacardium occidentale*	*Anacardaceae*	Kasu	Cashew nut tree	Bark, leaves
5	*Ananascomosus*	*Bromeliaceae*	Ope-Oyinbo Ehin-ahun Ekunkun	Pineapple	Unripe Fruit
6	*Azadirachtaindica*	*Meliaceae*	Dogonyaro	Neem	Bark, leaves
7	*Brideliaferruginea*	*Euphorbiaceae*	Ira odan		Bark, leaves
8	*Canna indica*	*Cannaceae*	Ido	Indian shot	Leaves
9	*Capsicum frutescens*	*Solanaceae*	Ata-Ijosi	Cayenne	Fruits
10	*Carica papaya*	*Caricaceae*	Ibepe	Pawpaw	Leaves, fruit
11	*Ceiba pentandra*	*Bombacaceae*	Araba	Kapok tree	Leaves

12	*Chromolaenaodorata*	*Compositae*	Ewe Akintola Ewe Awolowo	Siam weed	Root, leaves
13	*Chrysophyllumalbidum*	*Sapotaceae*	Agbalumo	African star apple	Bark, leaves
14	*Citrus aurantifolia*	*Rutaceae*	Osanwewe	Lime	Root, bark, stem-twigs, leaves, fruit
15	*Citrus aurantium*	*Rutaceae*	Osan ganinganin	Sour lime	Root, bark, stem-twigs, leaves, fruit.
16	*Citrus paradisi*	*Rutaceae*	Osan gerepu	Grape	Fruit, stem-twigs, leaves, root
17	*Curcuma longa*	*Zingiberaceae*	Laali-pupa	Turmeric	Rhizome
18	*Cymbopogon citratus*	*Poaceae*	Kooko-Oba	Lemon grass	Leaves
19	*Diospyros mespiliformis*	*Ebeneceae*	Igidudu	Ebony tree	Bark, leaves
20	*Enantiachlorantia*	*Annonaceae*	Osopa Awopa Dokitaigbo	African yellow wood	Bark
21	*Funtumiaafricana*	*Apocynaceae*	Ako-ire	Funtumia	Root
22	*Gossypium barbadense*	*Malvaceae*	Owu	Cotton	Leaves
23	*Gossypium hirsutum*	*Malvaceae*	Ela owu	Cotton	Leaves
24	*Harungana madagascariensis*	*Hypericaceae*	Asunje	Dragons blood tree	Bark, leaves
25	*Heliotropiumindicum*	*Boraginaceae*	Ogberi-akuko	Heliotrope	Whole plant.
26	*Hyptissuaveolens*	*Labiatae*	Jogbo		Leaves
27	*Khaya grandifoliola*	*Meliaceae*	Oganwo	Mahogany	Bark
28	*Lecaniodiscus cupanioides*	*Sapindaceae*	Akika		Roots
29	*Mangiferaindica*	*Anacardiaceae*	Mangoro	Mango	Bark, leaves
30	*Meliciaexcelsa*	*Moraceae*	Iroko	Iroko	Root, Bark
31	*Mondiawhitei*	*Periplocaceae*	Isirigun		Root, whole plant
32	*Morinda lucida*	*Rubiaceae*	Oruwo	Brimstone tree	Bark, leaves
33	*Musa sapientum*	*Musaceae*	Ogede were ibile	Banana	Fruits
34	*Nauclealatifolia*	*Rubiaceae*	Egberesi Gberesi	African peach	Root, bark, leaves
35	*Ocimumgratissimum*	*Labiatae*	Efirin-nla	Tea bush	Leaves
36	*Parquetinanigrescens*	*Periplocaceae*	Ogbo		Whole plant, leaves
37	*Pergulariadaemia*	*Asclepiadaceae*	Atufa, isirigun		Root, leaves
38	*Physalis angulata*	*Solanaceae*	Koropo		Leaves, whole plant
39	*Psidium guajava*	*Myrtaceae*	Gilofa	Guava	Bark, leave
40	*Pycnanthusangolensis*	*Myristicaceae*	Akomu		Bark
41	*Rauvolfia vomitoria*	*Apocynaceae*	Asofeyeje		Roots, barks, leaves
42	*Senna podocarpa*	*Caesalpiniaceae*	Asunwonibile		Bark, leaves
43	*Senna siamea*	*Caesalpiniaceae*	Kasia	Senna	Bark
44	*Solanum nigrum*	*Solanaceae*	Odu		Leaves
45	*Sphenocentrum jollyanum*	*Menispermaceae*	Akerejupon		Roots
46	*Tithoniadiversifolia*	*Compositae*	Jogbo Agbale	Tree marigold	Leaves, stem twings
47	*Tremaorientalis*	*Ulmaceae*	Afefe		Leaves, bark
48	*Vernonia amygdalina*	*Compositae*	Ewuro	Bitter leaf	Leaves
49	*Xylopia aethiopica*	*Annonaceae*	Erinje Eeru		Fruits, bark, leaves.
50	*Zingiber officinale*	*Zingiberaceae*	Ajo, Ata-ile	Ginger	Rhizome

Table 2. List of the formulation of some herbal mixture

Recipes containing two plants	Recipes containing three plants	Recipes containing four plants	Recipes containing five or more plants
Recipe 1 *Alstoniaboonei* (bark) *EnantiaChlorantha* (bark) Recipe 2 *Enantiachlorantha* (bark) *Curcuma longa* (rhizome) Recipe 3 *Citrus aurantifolia* (leaves, fruit) *Chrysophyllumalbidum* (leaves, bark) Recipe 4 *Capsicum fructescens* (fruit) *Alstoniaboonei* (bark) Recipe 5 *Citrus aurantium* (fruit) *Vernonia amygdalina* (leaves) Recipe 6 *Lecaniosdiscuscupanoides* (root) *Citrus aurantium* (fruit) Recipe 7 *Psidium guajava* (leaves) *Carica papaya* (leaves) Recipe 8 Gossypium barbadense *(leaves)* *Citrus aurantium* (fruit)	Recipe 1 *Diospyros mespiliformis* (bark) *EnantiaChlorantha* (bark) *Alstoniaboonei* (bark) Recipe 2 *Carica papaya* (fruit), *Citrus paradisi* (fruit) *Ananascomosus* (fruit) Recipe 3 *Physallisangulata* (leaves) *Tithoniadiversifolia* (leaves), *ChromolaenaOdorata* (leaves) Recipe 4 *Ocimumgratissimum* (Leaves), *Gossypium barbadense* (leaves) *Citrus aurantium* (fruit) Recipe 5 *Citrus aurantifolia* (leaves) *Curcuma longa* (rhizome) *Cymbopogon citratus* (leaves) Recipe 6 *Enantiachlorantha* (bark), *Funtumiaafricana* (root) *Zanthoxylumzanthoxyloides* (root)	Recipe 1 *Mangiferaindica* (bark, leaves), *Carica papaya* (leaves) *Alstoniaboonei* (bark) *Psidium guajava* (leaves) Recipe 2 *Ocimumgratissimum* (Leaves), *Vernonia amygdalina* (leaves), *Cymbopogon citratus* (leaves) *Azadirachtaindica* (bark, leaves) Recipe 3 Ananascomosus*(fruit)*, *Canna indica* (leaves) *Citrus aurantifolia* (fruit), *Citrus paradisi* (fruit)	Recipe 1 *Chrysophyllumalbidum* (leaves) *Citrus aurantifolia* (leaves) *Mangiferaindica* (bark, foliage leaves), *Sorghum bicolor* (leaves, stem) Anarcadiumoccidentale (bark) Recipe 2 *Psidium guajava* (bark, leaves) *Mangiferaindica* (bark, leaves), *Rauvolfia vomitoria* (bark, leaves) *Enanthiachlorantha* (bark), *Harunganamadagascariensis* (bark, leaves) *Curcuma longa* (rhizome) Recipe 3 *Curcuma longa* (foliage leaves), *Ocimumgratissimum* (Leaves) *Lecaniodiscuscupanioides* (foliage leaves) *Citrus aurantifolia* (foliage leaves), *Anarcadiumoccidentale* (foliage leaves) Recipe 4 *Lawsoniaguineensis* (leaves), *Citrus aurantifolia* (twigs, leaves, fruit) *Cymbopogon citratus* (leaves) *Carica papaya* (root) *Citrus aurantium* (fruit), Sphenocentrumjollyanum*(root)*

Table 2. List of Nigerian medicinal plants with antiviral properties

S/N	Botanical Name	Commonname	Family	Parts used
1	*Sida cordifolia*	Isankotu in Yoruba	Malvaceae	Whole plants
2	*EchinaceaPurpurea*	Dagumo/asofeyeje in Yoruba, Kashinyaro in Hausa, Yawo in Fulani	Asteraceae	Leaves
3	*Boerhaviadiffusa*	Etiponla, olowojeja in Yoruba	Nyctaginaceae	Roots
4	*Phyllanthus amarus*	Oyomokeisoamankedem" in Efik, "IyinOlobe" in Yoruba and "Ebebenizo" in Bini	Euphorbiaceae	leaves
5	*Andrographis paniculata*	Ewe-epa in yoruba	Acanthaceae	leaves
6	*Astragalus membranaceus*	Shekanbera" in Hausa and "aluki	Fabaceae	roots
7	*Borreriaverticillata*	Hausa: damfark'ami, Yoruba: irawo-ile	Rubiaceae	Whole plants
8.	Licorice (*Glycyrrhiza glabra*)	Ewe omisinmisin in Yoruba ,asukimaizaki in Hausa and Telugu in Igbo	Leguminosae	roots
9	Sage plants (*Salvia officinalis* L.)	Egbogi in Yoruba	Lamiaceae	leaves

5.2. Nigerian medicinal plants with antiviral properties

Nigerian plants have been shown to house a number of novel compounds with antiviral activities (figure 3). A number of scientific researches have elucidated the curative mechanisms by which these plants provide their therapeutic actions, while clinical research has presented the ability of some medicinal plants in treating many viral infections and diseases. For instance, *Sida cordifolia* has been reported to be a natural anti-human immunodeficiency virus (HIV) agent (Tamura *et al.,* 2010). One of the active compound isolated from the plant is (10E, 12Z)-9-hydroxyoctadeca-10,12-dienoic acid, a hydroxyl unsaturated fatty acid was found to be an exceptional NES (nuclear export signal) non-antagonistic inhibitor for nuclear export of Rev. Replication of HIV-1 is essentially dependent on the regulatory protein Rev or the Rev protein. Rev protein is involved in the nucleus-cytoplasm export of mRNA, which is very essential for the synthesis of the viral proteins necessary for viral replication. *Sida cordifolia* has also been proven to act as an immune booster serving as immune stimulants to strengthen and harmonize degenerative body systems and assist the immune system in its fight against invading antigens (bacteria and viruses) (Odukoya *et al.,* 2007).

Another plant with potent antiviral activities is *Boerhavia diffusa*. Active compound isolated from *Boerhaavia diffusa* extract is a glycoprotein with a molecular weight between 16,000 and 20,000. The protein and carbohydrates component of the glycoprotein is about 8 to 13 % and 70 to 80% is its composition respectively (Verma and Awatshi, 1979; Awasthi and Menzel, 1986). Other compounds whose biological activity with antiviral properties have been discovered from the plant include: boeravinone, Punarnavine, punarnavoside, hypoxanthine 9-L-arabinofuranoside, liirodendrin and ursolic acid (Lami *et al.,* 1992). Recipes from this plant alone or in combination with other medicinal plants show appreciable antiviral effects against many viruses which cause infections of the respiratory tract, liver and heart diseases. Obviously, there is no uniform principle of action against RNA viruses. Experimental findings on inhibition showed intense and broad antiphytoviral activity which suggested the mode of action of the glycoprotein inhibitor in medicinal plants. This causes a significantly effective antiviral drug candidtate to be synthesized in the plant cells, which then offers protection against viral infections (Verma and Awatshi, 1979).

Scientific literatures on *Echinacea* species have shown its health benefits with special focus often on immunological effects based on in vitro and in vivo studies. *Echinacea* and its preparations exert immune stimulant activity through three mechanisms: activation of phagocytosis, stimulation of fibroblasts, and the enhancement of respiratory activity that results in augmentation of leukocyte mobility. The production of cytokines (interleukin-1 (IL-1), IL-10) and tumour necrosis factor-α (TNF-α) is stimulated by *Echinacea purpurea* (Burger *et al.,* 1997). Some in vitro studies have proved the antiviral activity of various different preparations of Echinacea. Direct antiviral activity of *Echinacea purpurea* radix was analysed by means of a plaque-reduction- assay. The assay showed that the extract caused a 100% plaque-reduction down to concentrations of 200 ug/ml. The glycoprotein-containing fractions exhibited antiviral activity and decreased plaques numbers by up to 80%. It was concluded that the glycoprotein-containing fractions of *Echinacea purpurea* root extracts are able to induce the secretion of Il-1, TNF a, and IFN a, j3. Futhermore, they are at least partially responsible for the antiviral activity of Fchinacea purpurea radix (Bodinet and Beusher, 1991).

Assessment of antiviral activities of *Phyllanthus* species have shown its extract were most effective when administered either simultaneously with the initiation of virus infection or post infection but not when given pre-infection, and this suggested that the extract may act at the early stage of infection such as during viral attachment and entry as well as viral replication. The evidence from aqueous extract showed strong activity against viruses like HSV1 and HSV2 in vero cells by a process called quantitative polymerase chain reaction (Tan *et al.,* 2013). Western blot and 2D-gel electrophoresis were used to examined protein expressions of treated and untreated infected vero cells. *Phyllanthus amarus* and *Phyllanthus urinaria* showed the strongest antiviral activity against both HSV1 and HSV2 viruses. Their therapeutic actions were proposed to be at the early stage of replication and infection (Tan *et al.,* 2013). The phytochemicals contributed to the antiviral activities of the plant include rutin, gallic acid, caffeolquinic acid, geraniin, corilagen, galloylglucopyranose, digalloylglucopyranoside, trigalloyglucopyranoside, quercetin glucoside and quercetin rhamnoside (Tang *et al.,* 2010; Lee *et al.,* 2011).

The antiviral activity of *Andrographis paniculata* (Burm. f.) extract was determined using Real Time – Polymerase Chain Reaction (RT-PCR) analysis to examine its ability to inhibit virus load in A549 cells transfected with Simian Retro Virus (SRV). The immune-stimulant activity of extract was determined by its ability to enhance lymphocytes cell proliferation using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The result of this study revealed that ethanol extract of *A. paniculata* inhibited the SRV virus titer and it was not toxic to the cell line. Also, *A. paniculata* extract at low concentration enhanced lymphocyte cell proliferation (Churiyah *et al.,* 2015). These results were also similar to that of Wiart *et al.* (2005) which demonstrated andrographolide viricidal activity against herpes simplex virus 1 (HSV-1) without significant cytotoxicity. Lin *et al.* (2008) also established that ethanol extract *A. paniculata* and andrographolide inhibited Epstein-Barr virus (EBV) lytic proteins during the viral lytic cycle in P3HR1 cells.

Astragalus membranaceus is the dry root of *Astragalus mongolicus* or *Membranous astragalus*. The active compounds isolated from *Astragalus membranaceus* are flavonoids, saponins, and polysaccharides (Kojo *et al.,* 2013). Previous studies showed that the *Astragalus membranaceus* injection showed obvious anti-influenza virus activity. It improved the survival rate of Raw264.7 cells which were infected with influenza virus, enhanced the blocking effect of influenza virus on cell cycle after infection, reduced the MDA content and increased the SOD activity. At the same time, the innate immunity was affected by regulating the expression of TLR3, TAK1, TBK1, IRF3, and IFN-β in the TLR3-mediated signalling pathway, thus exerting its antiviral effect in vitro (Yuxi *et al.,* 2019).

Borreira are genera of *Rubiaceae* widespread in tropical and subtropical Africa. Some species of these genera perform crucial functions in herbal and traditional medicine in Europe, Africa South America, Asia. They are used in the treatment and management of diseases such as respiratory infections, inflammation of eye, malaria, skin diseases, fever, hemorrhage, urinary infections, diarrheal and other digestive problems, headache, and gums. Different biological activities such as antioxidant, antimicrobial, anti-inflammatory, antitumor, anti-ulcer, larvicidal, gastrointestinal, and hepatoprotective have been reported from various extracts *Borreira*. These biological activities have been attributed bioactive compounds from the plant such as terpenoids, flavonoids, with alkaloids and iridoids as the major active principles (Lucia and Jesu, 2012). Phytochemical screening has shown the presence of alkaloid called emetine in *B. verticillata* (Moreira, 1964). And this emetine has been reported to have antiviral effect against SARS-CoV-2 virus in Vero E6 cells with the estimated 50% effective concentration at 0.46 μM when tested alongside with Remdesivir, lopinavir and homoharringtonine in their capacity to inhibit SARS-CoV-2 replication in vitro. Results have also shown that emetine, an anti-protozoal agent, potently inhibits ZIKV and EBOV infection with a low nanomolar half maximal inhibitory concentration (IC50) *in vitro* and potent activity in vivo. Two mechanisms of action for emetine were also identified which are inhibition of ZIKV NS5 polymerase activity and disruption of lysosomal function. Emetine also inhibits EBOV entry (Yang *et al.,* 2018).

It was reported that the Licorice root has been used for ages in ancient Egyptian medicine and also in Indian Ayurvedic medicine and also in traditional Chinese medicine. Licorice root had already been known for its antiviral properties, but researchers noticed that during the SARS outbreak certain groups of people drinking concoctions of traditional Chinese medicine that contained it during the SARS outbreak did not get infected with the virus despite having been exposed to it by having relatives in the same household who were infected. It was this that triggered the initial research (Jakkapong, 2020). The licorice root contains a variety of phytochemicals such as flavonoids like Glycyrrhizin, Liquiritigenin, and Glabridin that also had antiviral activity against the SARS coronavirus. But it is the the class of phytochemicals called triterpenoids also found in licorice root especially glycyrrhizic acid and glycyrrhetinic acid that was found to be extremely potent against the SARS coronavirus (Gerold *et al.,* 2005).

Glycyrrhizin affects cellular signaling pathways such as protein kinase C; casein kinase II; and transcription factors such as activator protein 1 and nuclear factor κB. Furthermore, glycyrrhizin and its aglycone metabolite 18β glycyrrhetinic acid upregulate expression of inducible nitrous oxide synthase and production of nitrous oxide in macrophages (Jeong and Kim, 2002). Preliminary results showed that glycyrrhizin induces nitrous oxide synthase in Vero cells to suppress virus replication (Cinat *et al.,* 2003).

Glycyrrhizin reduced concentrations of P24 antigen and upregulation of chemokines in patients with HIV-1 and chronic hepatitis C virus. However, infrequent side-effects such as increase in blood pressure and hypokalaemia were documented in some patients after many months of glycyrrhizin treatment (Booth *et al.,* 2003).

5.3. Nigerian medicinal plants with anti-inflammatory properties

Inflammation has been implicated in the pathogenesis of COVID-19. It is a physiological/defense response of the host to harmful stimuli such as pathogenic infections, allergens, chemical toxicity or injury to the tissues. If left uncontrolled, inflammatory response can result into deleterious effects such as cancer, cardiovascular dysfunctions, autoimmune diseases and metabolic syndrome (Bagad *et al.,* 2013). Modern medicines like non-steroid anti-inflammatory drugs, steroids, and immunosuppressant have been used to control and suppress inflammatory diseases but with associated unwanted side effects. Thus, the need for natural anti-inflammatory agents with increased pharmacological response and no or lowest degree of adverse effects (Bagad *et al.,* 2013; Oladele *et al.,* 2017) which is obtainable in medicinal plants. *Curcuma longa* (Turmeric) is one of such medicinal plants, the most essential metabolite of turmeric is curcumin and its responsible for its anti-inflammatory properties (Jurenka, 2009; Oladele *et al.,* 2020a). Many clinical studies have been carried out to prove the anti-inflammatory effect of curcumin in diseases such as rheumatoid arthritis and reduced its clinical manifestation such as joint swelling and morning stiffness when compared with standard drug, phenylbutazone (Deodhar *et al.,* 1980). Curcumin also offers beneficial effects in treatment of ulcerative colitis (Hanai *et al.,* 2006), irritable bowel syndrome (IBS) (Bundy *et al.,* 2004), psoriasis (by the selective prohibition of phosphorylase kinase) (Heng *et al.,* 2000) and acts as a reducing agent to delayed graft rejection (DGR) after kidney transplant surgery (Shoskes *et al.,* 2005).

Similarly, *Zingiber officinale* (ginger) has been shown to have potent anti-inflammatory effects. Ginger powder has had ameliorative effect in musculoskeletal and rheumatism patients through inhibiting cyclooxygenase and lipoxygenase pathway in synovial fluid (Srivastava and Mustafa, 1992). Shimoda *et al.* (2010) reported the anti-inflammatory potential of *Zingiber officinale* in acute and chronic inflammation models. The result showed that ginger possesses effective inhibitory effects on acute and chronic inflammation, and suppressed activation of macrophage via anti-inflammatory pathway. *Zingiber officinale* have been reported to decrease serum level of TNF-α and high-sensitivity C-reactive protein (hs-CRP) in type 2 diabetic patients (Mahluji *et al.,* 2013). Other medicinal plants with anti-inflammatory properties that could offer protection against coronavirus-induced inflammation include *Combretum mucronatum, Ficus iteophylla, Moringa oliefera (Moringaceae), Schwenkia americana, Alafia barteri, Dichrostachys cinerea, Capparis thoningii Schum, Cassia occidentalis (Caesalpiniaceae), Asparagus africanus,* and *Indigofera pulchra.*

5.4. Nigerian medicinal plants with immune-boosting properties

Survival of homo sapiens against traumas from foreign pathogenic microorganisms depend on the status of their immune defense mechanisms. It is well established that the immune system safeguards the host against attacks from infective microorganism such as virus, allergic or toxic molecules (chaplin, 2010). Once a defect occurs within the

immune system, it results in response impairment against infectious agents. The cause of impairment (immunosuppression) can be either intrinsic (inherited) or extrinsic and referred to as primary or secondary immunodeficiency, respectively (Abbas *et al.,* 2016; Chinen and Shearer, 2010).

Immunomodulatory agents are non-specific compounds that work without antigenic specificity similar to the adjuvants that are associated with some vaccines (Gupta *et al.,* 2010; Liu *et al.,* 2016). Medicinal plants and natural products with immunomodulatory activities have been employed in traditional medicine and phytomedicine. They improve the humoral and cell-mediated immunity and mediate the initiation of "non-specific" immune responses which include the induction of macrophages, natural killer cells, granulocytes, and the complement system. These processes trigger the synthesis and release of diverse molecules such as cytokines which participate in the improvement and modulation of the immune responses (Gummet *et al.,* 1999; Vigila and Baskaran, 2008). Put together, the entire series of reactions serves as a substitute for the present chemotherapy for immunodeficiency offering protection against infections caused by various pathogenic agents (Sultana *et al.,* 2011).

Plants that are copious carotenoids, vitamin C or flavonoids can act as an immune-stimulant. Medicinal plants that are rich in flavonoids may also possess anti-inflammatory action. They induce interferon production, enhance the activity of lymphocytes and increase phagocytosis. Examples of these immunomodulatory plants include garlic which remarkably enhance immune system activities. Garlic as an immune system booster has been found to exert an immune-potentiating effect by stimulating natural killer cell activity. Some studies strongly present garlic as a promising candidate as an immune modifier, which preserves the homeostasis of immune functions (Kyo *et al.,* 2001) because it has a higher concentration of sulfur combinations which are responsible for its therapeutic effects.

Other medicinal plants used by traditional healers as immune-boosters are garlic (*Allium sativum*), guava leave (*Psidium guajava*), lemongrass (*Cymbopogon citratus*), cinnamon (*Cinnamomum zeylanicum*).

5.5. Nigerian medicinal plants with antioxidant properties

Numerous plants that grow in Nigeria are well-known to have countless therapeutic potentials that could be due to their antioxidant properties (Oladele *et al.,* 2020b,c). Plants are known to be the main source of natural antioxidants in the form of phenolic compounds (phenolic acids, flavonoids and polyphenols). Most of the anti-inflammatory, digestive, neuroprotective, hepatoprotective and nephroprotective drugs derived from natural origin have been reported to have antioxidant/radical scavenging mechanism as part of their activity (Oladele *et al.,* 2020b,c; Oyewole *et al.,* 2017). The ingestion of natural antioxidants has been associated with the reduced risk of cancer, cardiovascular disease, diabetes and other diseases associated with ageing (Ajayi *et al.,* 2017). Hence, interest has been increased for finding antioxidants of plant source, which are safe and suitable for use in food and/or medicine. In that regard, due to the increasing numbers of diseases ravaging the continent of Africa and of course the world at large, there has been an increased interest in finding antioxidants from plant source, which are safe and suitable for use in food and/or medicine. For that reason, many indigenous plants were selected for their significant antioxidant activities.

5.5.1 Antioxidants from plants

The use of artificial and natural food antioxidants regularly in medicine and foods particularly those having fats and oils to shield the food from oxidation. Butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) are some of those artificial natural food antioxidants which have been used expansively in food, cosmetics, and in many healing products industries. Nevertheless, due to the effect of high temperatures which bring instability in them, high volatility, synthetic antioxidant's carcinogenic behaviour, users dispositions steered to shift in respect to the producers or manufacturers from man-made to natural antioxidants (Papas, 1999).

Considering the increasing numbers of risk issues of humans to various harmful diseases, this brought about the need for the use of natural constituents present in dietary and medicinal plants as curative and helpful antioxidants. A vast number of indigenous plants in Nigeria has been reported to reveal antioxidant activity, including *Allium sativum, Zingiber officinale, Crocus sativus, Dodonaea viscose, Barlerianoctiflora, Anacardium occidentale, Datura fastuosa, Caesalpiniabonducella* and many more as in table 3. Several of these antioxidants from plants has been shown to be an active oxygen scavengers or free radical, with has no harmful effects on human body (El makawy *et al.,* 2020). For detrimental effects of reactive oxygen species to be stopped, plants have shown a powerfully in-built enzymatic and non-enzymatic scavenging capacity. These Enzymes included dehydroascorbate reductase (DHAR), catalase (CAT), glutathione S-transferase (GST), superoxide dismutase (SOD), glutathione peroxidase (GPX), ascorbate peroxidase (APX), glutathione reductase (GR), monodehydroascorbate reductase (MDHAR) and peroxidases (POX), while non-enzymatic compounds include tocopherols, ascorbate (AsA), glutathione (GSH), and carotenoids.. In plants, any form of rise in the levels of antioxidants has been established to exhibit a better resistance to different types of environmental stresses (Hasanuzzaman *et al.,* 2011).

Table 3. List of medicinal plants with antioxidant properties

S/N	Name of plants	Common English Name	Nigerian names	Family	Plant part used
1	*Zingiber officinale*, Ginger Zingiberaceae Rhiz□me Tohma *et al.*, 2017	Ginger	Atale in yoruba	Zingiberaceae	Rhizome
2	Cucurma longa	Turmeric	Gangamau (Hausa) or Atale pupa (Yoruba) or Boboch (Igbo)	Zingiberaceae	Rhizome
3	*Allium sativum* Garlic Alliaceae Bulb Elost	Garlic	Aayu in Yoruba Ayo-ishi in Igbo and Tafarunua in Hausa	Alliaceae	Bulb
4	*Cannabis sativa L*	Hemp/marijuana	Igbo in Yoruba	cannabaceae	seed
5	Ageratum conyzoides	Goat weed	Imi esu in Yoruba	asteraceae	leaves
6	Ficus exasperata	Ficus plants	Epin in Yoruba	Moraceae	leaves
7	Telifarria occidentalis	Fluted plumkin	Ugwu in Igbo	Curcurbitaceae	leaves
8	Vernonia amygdalina	Bitter leaves	Ewuro in Yoruba ,		Leaves
9	*Garcinia kola*	Bitter kola	Orogbo in Yoruba, Adu/aku-inu and Namiji goro in Hausa	Clusiaceae	*seeds*
10	Ocimum basillicum	Basil/ scent leaf	Efirin in Yoruba Nchanwu in Igbo and Dadoya in Hausa	Lamiaceae	leaves
11	Psidium guajava	guava	Psidium	Myrtaceae	*leaves*
12	xylopia aethiopica	Ethiopian pepper	Eeru/ Erunje in Yoruba, Uda in Igbo and Kimba in Hausa	Annonaceae	seeds
13	Parkia bigglobossa	Locust bean	Iru in Yoruba, ogiri in Igbo and dadawa in Hausa	Papilionacea	seeds
14	Spondia mombi	hogplum	Iyeye in Yoruba,	Anacardiaceae	leaves
15	Musa paradisiaca)	Plantain	Ogede Agbagbain Yoruba, Abrika in Igbo and ayaba in Hausa		fruits
16	Azadirachta indica	Neem tree	Dongoyaro in Yoruba, Atu yabasi / ogwu akom in Igbo and Maina in Hausa	Meliaceae	leaves

5.5.2 Antioxidants from fruits and vegetables

Consistent eating of dietary foods like fruits and vegetables is well documented to have potential in the management of various chronic ailments affecting human in Africa. These supplements of antioxidant are directly being obtained from fresh fruits and vegetables, which contain a vast quantity of alkaloids, flavonoids and antioxidant complements which can take part in the defense mechanisms against different cardiovascular ailments including different types of cancers and many health problems (Oladele *et al.,* 2020a,b; Ajayi *et al.,* 2017).

Documented reports have revealed that a diet with rich antioxidants has an great impact on health in many ways that vast variety of plants and plant parts have been established to contain a large amount of antioxidants such as strawberries, blueberries, grapes, spinach, plums, broccoli flowers, alfalfa sprouts and many more with antiviral properties. Citrus fruits like lemons, oranges etc. also contain a high quantity of natural antioxidants, most significantly vitamin C (Al-snafi, 2015; Oladele *et al.,* 2020d).

However, there are some new and unique antioxidants like derivatives of flavonoids and p-coumaric acid that have been found in spinach. NAO- a spinach-derived natural antioxidant that contains derivatives of flavonoids and p-coumaric acid playing a significant role in the prevention of prostate cancer. Recently, it has also been established that fruits like araticudomato, pindo palm and jackfruit are good sources of vitamin C, vitamin A and other phenolic compounds; analysis on these fruits is being carried out to create the genetic, chemical or biological variations so as to enhance the antioxidant potential of the same (Shebis *et al.,* 2013; Oladele *et al.,* 2020d).

5.5.3 Neem

Neem (*Azadirachta indica*) as it is fondly called is a tree inside the family of *Meliaceae*. Neem is also known as 'Dongoyaro in Yoruba, Atuyabasi /ogwuakom in Igbo and Maina in Hausa word. Seeds, oil, roots, roots, bark, leaves and seeds as parts of the tree are somehow bitter and contain compounds with a proven potential as an anti-inflammatory, anti-ulcer and antifungal, antiviral, antiplasmiodal, cytotoxic, antipyretic anti-microbial and antiseptic in nature (Emran *et al.,* 2015). The incorporated chemical constituents with many biologically energetic compounds that can be extracted from neem include flavonoids, alkaloids, carotenoids, triterpenoids, phenolic compounds, steroids and ketones. Azadirachtin is validly an incorporation of seven isomeric compounds which was labelled as azadirachtin A-G and azadirachtin E is more efficient (Verkerk and Wright, 1993).

5.5.4 Ginger

Ginger (*Zingiber officinale* Roscoe) is an important tropical valued medicinal herb that is found globally as a spice and used for healing and therapeutic proposes. Ginger belongs to the *Zingiberaceae* family which has been reported to contain over 1250 species in fifty genera, together with 4 other families which is positioned in the order Zingiberales and class Monocotyledones (Berg, 1997). It has been reported to have many vital pharmacological actions to treat various types of diseases by the actions of antiemetic, antioxidant, anti-cancer, anticoagulant property, anti-inflammatory, and soon. Clinical studies have documented its efficacy in treatment of post-operative vomiting and vomiting of pregnancy.

The pungency characteristics of ginger are said to be due to gingerols and shogaols found in them. It has been established that the main components of ginger are the aromatic essential oils, the antioxidants and the pungent oleo-resin. Pungent compound has been identified as the phenylalkylketones, known as gingerols, shogaols, and zingerone (Rajesh and Subha, 2018). All main active constituents of *Zingiber officinale* Roscoe, such as zingerone, gingerdiol, zingibrene, gingerols and shogaols, have been proven to have anti-oxidant activities (Chrubasik *et al.,* 2005), and this antioxidant activity in ginger is due to the existence of polyphenol compounds like (6-gingerol and its derivatives). The main active constituents of ginger are Volatile oil (zingiberene, curcumene, farnesene, zingiberol, D-camphor), Shogaols, Diarylheptanoids, Gingerols, Paradol, Zerumbone, 1-Dehydro-(10) gingerdione, Terpenoids and Ginger flavonoids (Baliga *et al.,* 2012); these compounds give ginger its characteristic hot sensation.

5.5.5 Ageratum conyzoides

The genus *Ageratum* is derived from the Greek words *'ageras'* meaning non-aging which refers to long life-time of plant, and the species epithet *'konyz'* is the Greek name of *Inula helenium* which resembles the plant. *Ageratum conyzoides* Linn with a Family Asteraceae is an annual herb with an extensive history of traditional medicinal use in the tropical and sub-tropical regions of the world, commonly known as Billy goat weeds. The stems and leaves of the plant are fully covered with fine white hairs (Adewole, 2002).

5.5.6 Monoterpenes and sesquiterpenes

About 51 constituents have been reported to have been obtained from the analysis of the Ageratum oil sample from Nigeria which makes it the highest to include 20 monoterpenes (6.6%) of which 1% contains sabinene, 1.6% contains β-pinene and β-phellandrene, 2.9% contains 1.8-cineole and limonene, 0.6% contains terpenen-4-ol and 0.5% contains α-terpineol and further found 20 sesquiterpenes (5.1%), and that of single substance were found to be in traces approximately 0.1%. Indian Ageratum oil from goat weed is found to contain 5.3% ocimene which was found in traces from Nigerian plant, 6.6% α-pinene, 4.4% eugenol and 1.8% methyleugenol (Rao and Nigam, 1973). The major sesquiterpenes are beta-caryophyllene, 1.9 to 10.5% from an oil sample obtained from Cameroon and 14 to 17% in a Pakistani oil sample. Another sesquiterpene, δ- cadinene occurred in approximately4.3% in the oil received from Indian plants (Rao and Nigam, 1973). Sesquiphellandrene and caryophylleneepoxide have been obtained in 1.2 and 0.5%, respectively from leaves (Ekundayo *et al.,* 1988). The plant has been examined on the basis of the scientific in vitro, in vivo or clinical evaluations to have possessed the major pharmacological activities that includes analgesic activity, antimicrobial activity, anti-inflammatory activity, spasmolytic effects, gamma radiation effects, anti-cancer and radical scavenging activity, antimalarial activity and others activities based on the listed bioactive earlier discussed (Singh *et al.,* 2013).

5.5.7 Guava

Guava has been reported to have a high number of antioxidants and anti-providing nutrients which are essential not only for proper functioning of life but also help to control the free radical activities. It also has a variety of phytochemicals which are useful for human health like diabetes, obesity and high blood pressure. There are two common methods by which antioxidants neutralize free radicals that are DPPH and FRAP assay. Extracts of guava in water and organic solvents have a large quantity of antioxidants which can stop the oxidation reaction. Pink guava also has a high antioxidant activity (Musa *et al.,* 2011). In fruits, the most abundant oxidants are polyphenols and ascorbic acid. The polyphenols are mostly flavonoids and are mainly present in glycoside and ester forms. The free elagic acid and glycosides of apigenin, myricetin, Quercetin, quercetin-3-O-glucopyranoside and morinare were found to be present in guava, and the presence of all these bioactives makes it a potent antioxidant (Nantitanon and Okonogi, 2012).

5.5.8 Allium sativum

Garlic is well accepted as a spice around the Africa continent and Nigeria, and it has been proven to act as a herbal remedy for the prevention and treatment of several diseases. It has also been reported to have an anti-bacterial, anti-viral, anti-protozoal, anti-cancer, anti-fungal, immunomodulatory, anti-inflammatory, hypoglycemic and

hypocholesterolemic potentials (Rehman and Munir, 2015), Allicin being the principal compound in aqueous garlic extract or raw garlic homogenate which is responsible for the cholesterol-lowering effect in humans and animals. When garlic bulb is crushed, the enzyme allinase activates alliin, a non-protein amino acid present in the intact garlic, to produce Allicin (Chowdhury *et al*., 2002). The phytochemical screening of garlic has also been reported to have chemical compounds such as saponin, tannin, carbohydrates, cardio glycoside, alkaloids, flavonoid, phlobatannin and glycoside (Pavni *et al*., 2011).

5.6 Nigerian medicinal plants that enhance membrane integrity

The antiviral mechanisms of different extracts preparations of many vegetal products have been through the disturbance of cell membrane integrity, thereby increasing the membrane permeability, and invariably causing the leakage of the RNA or DNA of the virus (Bouyahya *et al.,* 2019), whereas fortifying or strengthening the cell membrane with nutraceuticals that offer protection to the integrity promises to be a functional approach to preventing invasion by known viruses and by extension the novel SARS-COV-2. Among the protective vegetal natural products reported to preserve or enhance cell membrane integrity are the following, some of which also have antiviral activities against respiratory viruses causing flu (gripe), while a few others have been studied against the earlier members of the coronavirus family such as the MERS and SARS. Many bioactive compounds from the vegetal sources have been shown to interact with the surface of cell membranes to prevent viral entry, specifically binding to membrane carrier proteins, regulating ion channels, modulating enzymes, influencing the order of the membrane lipid bilateral to elicit their medicinal activities. While there exists a plethora of membrane-modulating bioactive vegetal components, nutraceuticals, and phytochemicals, a variety of peptides are also secreted by plants with lipophilic properties that enhance their ability to pass across cell membranes (Tsuchiya, 2015). Many of these structural compounds have been studied to decipher their mechanistic transportation across cellular, intracellular, and artificial membranes, as well as their effects on gene expression within the nucleus following possible participation in signalling pathways. Many of the structure-activity relationships have often been described by several authors vis-a-vis how they affect the fluidity, micro-viscosity, order, elasticity, and permeability of both biological and artificial membranes.

Among these are *Allium cepa* of the Amaryllidaceae family, rich in quercetin, which inhibits the SARS main proteases, 3CLpro and PLpro, and the Middle Eastern Respiratory Syndrome virus (MERS) 3CLpro protease, *in vitro* (Mani *et al.,* 2020). It has also been proposed that the modulation of cellular unfolded protein response (UPR) and autophagy signalling being important to coronaviruses to complete different stages of the viral life cycle during infection, if perturbed by quercetin and resveratrol through the mitochondrial permeability transition pores (MPTP) and NLRP-3 inflammasome pathways, may have anti-coronavirus effects (Nabirotchkin *et al.,* 2020). *Artemisia annua* (qinghao) is a plant of the Asteraceae family from which artemisinin is extracted. Together with its derivative, dihydroartemisinin, it has shown promise against parasites and viruses, including the human cytomegalovirus, *in vitro* (Flobinus *et al.,* 2014). The plant has also shown potent anti-HIV (Lubbe *et al.,* 2012), and anti–SARS-CoV effects (Li *et al.,* 2005). The leaf and bark of *Azadirachta indica* L belonging to the family *Meliaceae* showed antiviral activity against herpes simplex virus type-1 infection as a potent entry inhibitor (Tiwari *et al.,* 2010). Some of its bioactive compounds also boost the immune system by upregulating polymorphonuclear (PMN) leukocytes, macrophage activity, and lymphocyte proliferation response (SaiRam *et al.,* 1997). The aqueous extract of the branches was found to be effective against the Newcastle disease virus in embryonated SPF chicken eggs and SPF chickens. The plant is known to be rich in salanin, nimbin, azadirone, and azadirachtins (Ong *et al.,* 2014) and show potent antiviral activities (Sarah *et al*., 2019).

Camellia sinensis of the family Theaceae is rich in catechins and flavonoids [epigallocatechingallate] (EGCG), epicatechin (EC), epigallocatechin (EGC) and epicatechin gallate (ECG)] (Baibado *et al.,* 2011), and alkaloids (caffeine, theobromine, theophylline. They are known as anti-inflammatory and antioxidant compounds (Mahmood *et al.,* 2016) that efficiently relieve chronic obstructive lung disease (COPD), while at the same time reducing the risk of lung cancer and type 2 diabetes, which can constitute serious underlying conditions that predispose to grave clinical outcomes for the SARS-COV-2. *Chamaemelum nobile* contains apigenin, a dietary flavonoid indicated for inflammation, cold, and asthma (Kim *et al.,* 2014) based on its antioxidant, anti-inflammatory, and properties (Cardenas *et al.,* 2016). The bark of *Cinchona officinalis* (quina-quina), *Rubiaceae* is rich in quinine ((8S, 9R)-6′-methoxycinchonana-9-ol; (αR)-α-(6-methoxy-4-quinoyl)-α-[(2S, 4S, 5R)-(5-vinylquinuclidin-20yl)] methanol), which has been in use for the treatment of malaria as far back as 1632 (Baird *et al.,* 1996). It was shown to have therapeutic effects against influenza virus infections in animal studies (Seeler *et al.,* 1946). *Cinnamon verum*, of the genus Cinnamomum (Family Lauraceae), contains proanthocyanidin and (epi)catechins. It is known to have antiviral, antibacterial, antioxidant, anti-inflammatory, and immunomodulatory properties (Kumar *et al.,* 2019; Polansky and Lori, 2020). Its extract has anti-RNA viral effects and inhibited the wild type SARS infection, *in vitro* possibly blocking cell entry via endocytosis (Zhuanga *et al.,* 2009). *Citrus aurantium /Sinensis* (Rutaceae) peel, containing hesperidin and vitamin C, has antioxidant and antiviral activities (M'hiri *et al.,* 2017). The flower extract of *Citrus aurantium* protected cardiomyocyte cell membrane in Isoproterenol pre-treated male rats (Keshtkar *et al.,* 2017). *Curcuma longa* (turmeric) contains curcumin which, like pterostilbene, interacts with the C-terminal of S1 domain with significant binding energies (Jitendra *et al.,* 2020). *Cymbopogon citratus* Stapf of the Poaceae family possesses anti-allergic property indicated for the treatment of asthma by limiting the infiltration of inflammatory cells into the lungs (Santos *et al.,* 2015).

Euphorbia hirta Linn. is a common plant used to treat asthma and other respiratory diseases including chronic flu, including asthma and bronchitis due to its anti-inflammatory and antiasthmatic activities (Kumar *et al.,* 2010). *Piper nigrum* is another plant whose seeds have been indicated for the treatment of pharyngitis arising from flu and viral infection (DeFilipps and Krupnick, 2018). The antiviral action has been attributed to the ability to fracture, disrupt, and completely collapses the plasma membrane of pathogens, thereby increasing cell permeabilization and disrupting membrane integrity (Zou *et al.,* 2015).

Fragaria ananassa of the rose family (Rosaceae) contains fisetin (3,3′,4′,7-tetrahydroxyflavone), a pigment flavonol is also abundant in grapes, apples, onions, and cucumbers. It is also a senolytic agent, as it selectively induces death of senescent cells to alleviate age-related diseases (Yousefzadeh *et al.,* 2018). Fisetin, quercetin, isorhamnetin, genistein, luteolin, resveratrol, and apigenin have been reported to interact with both S 1 and S2 domains of the spike protein of SARS-COV-2 with appreciable binding energies thus disrupting viral attachment and internalization into the host (Jitendra *et al.,* 2020).

Garcinia kola Heckel (Clusiaceae), known to contain the biflavonoid kolaviron, is popular for the treatment of malaria, hepatitis, neurodegenerative disease, male sexual dysfunction, and immune-destructive diseases (Farombi *et al.,* 2019; Uko *et al.,* 2001). It also protects against the oxidation of lipoprotein (Farombi *et al.,* 2008). *Garcinia kola* is also used to relieve cold and cure laryngitis (Manourova *et al.,* 2019). *Phyllanthus emblica L. (Euphorbiaceae),* contains appreciably small molecular weight tetra-O-galloyl-β-d-glucose, an anti-HBV bioactive compound (Xiang *et al.,* 2010), while *Eclipta prostrata* L. (Asteraceae) as well as peanut shells, green leafy vegetables such as spinach contain the anti-inflammatory and antioxidant luteolin (Arunachalam *et al.,* 2009). These two bioactive compounds were reported to be able to bind strongly to the S-protein of SARS-CoV. By this mechanism, they were able to delay or prevent viral entry into host cells via the membrane receptors (Yi *et al.,* 2004).

Zanthoxylum zanthoxyloides Lam. (Rutaceae), contains tortozanthoxylamide (N-(isobutyl) 3, 4-methylenedioxy cinnamoyl amide) (Dofuor *et al.,* 2019) which has anti-inflammatory, antitrypanosomal and antispasmodic properties (Guendehou *et al.,* 2018). *Zingiber officinale* Roscoe, of the Zingiberaceae family, contains gingerols which showed antiviral properties against the human respiratory syncytial virus on HEp2 and A549 cell line (Chang *et al.,* 2013). The anti-inflammatory and antioxidant properties have also been described in dopaminergic neurons in Parkinson's disease models (Park *et al.,* 2013), and other cell types. Oleoresin, gingerol, shogaol, and zingerone from ginger increased the percentage of CD3+CD4+ thus improving cellular and humoral immune response in HIV patients (Tejasari, 2007). Ginger also alleviated bronchopulmonary dysplasia and inflammation induced by hyperoxia and intrauterine LPS in a chorioamnionitis rat model (Cifci *et al.,* 2018).

Many of these vegetal products promote bronchodilation, thus relieving fluid in the lungs, preventing lung fibrosis or plague formation, naso-/trachea-pharyngeal clogging, alleviating (dry) cough, chest pain, and difficulty in breathing. Overall, these novel chemical entities can be explored as formulations or cocktail containing promising druggable agents against the novel SARS-CoV-2 for blocking receptor binding and/or viral phagocytotic internalization of the SARS-CoV-2 following the binding of the S- (spike) protein to the angiotensin-converting enzyme 2 (ACE-2) receptors, and its associated proteases such as the transmembrane protease serine 2 (TMPRSS2), Cathepsin L (CTSL) and Cathepsin B (CTSB), which are ubiquitously present in almost all of the human cells, the existence of which does not indicate that the virus can always infect the cells that express them.

5.7 Nigerian medicinal plants used in the treatment of respiratory infections, cough, and flu

Phytochemical-based treatments for respiratory infections and related syndromes have been in use in many nations in Africa for many decades (table 4). Respiratory infections particularly pneumonia, asthma, tuberculosis, sinusitis, and rhinitis represent the main factors of morbidity and mortality in both developed and developing nations of the world (Ait-Khaled *et al.,* 2007).

Table 4. Nigerian recipes for the treatment of respiratory infections, cough and flu

Recipe 1. Containing eight plants	Recipe 2. Containing sixteen plant materials
Tetrapleura tetraptera Leguminosae bark, root and leaf	Canarium scheweinfurthii called Awogba or Gbogbonise in Yoruba (part to use - Root)
Azadirachta indica A. Juss (Meliaceae) bark, root and leaf	Axonopus compessus (Poaceae) Guil & Perr. (Part to use - Stem)
Heliotropium indicum Linn. (Boraginaceae) root	Anogeissus leiocarpus, (D.C) Guil.L. & per. (Part to use -Stem).
Opuntia dillenii Haw (Cactaceae)	Capsicum annum, Linn., called Ata Ibile in yoruba (part to use -Fruit).
Khaya grandifoliola (Meliaceae)	Curculigo pilosaq, Schum.& Thonn, Engl., called Epakun in yoruba (part to use Rhyzome)
Allium sativum	Gladiolus daleni, Van. Geal called Isu Baka in Yoruba. (part to use -Rhyzome)
Capsicum frutescens	Allium ascalonicum, Linn. called Alubosa Elewe in Yoruba (part to use- Bulb)
Turmeric	Dalbergienna welwitshii, white and red species, Bal (part to use- Root)
	Solanum luberosum, L.cv called isu arinda in yoruba(part to use- tuber)
	Sphenostylis stenocarpa called Ewa sese in yoruba (beans)
	Vigna sinensi called ewa ikakure in yoruba (beans)
	Khaya grandifoliola,C.D.C (part to use - bark)
	Anhodersta djalonensis, A. Cher., called Sapo in Yoruba (part to use -bark).
	Cassia alata also called Ringwork plant is called Asunrin dudu in yoruba (root)
	Citrullus colocynthis, Linn Schard, called bara in Yoruba (fruit).
	Camphor called kafura pelebe in Yoruba Iseta (part used root).
	Local substitute salt called Obuotoyo in Yoruba,

Anogeissus leiocarpa belonging to the family of *Combretaceae* is also called "Idi Ayin" among the Yoruba people of Nigeria. It is a deciduous plant indigenous to the savannas of tropical Africa. It is also referred to as African birch. *A. leiocarpa's* root and bark are used traditionally in the treatment of cough, gonorrhea, asthma, tuberculosis.

Allium sativim belonging to the Amaryllidaceae family, known as Aayu among the Yoruba people is also popularly called garlic among the three tribes of Nigeria. It is being used as a food supplement and in folklore medicine for several centuries; it is the most researched medicinal plant (Milner, 1996). Garlic has been used useful to the treatment of a wide range of diseases such as coronary heart disease, high blood pressure, heart attack, high cholesterol, and hardening of the arteries due to its biologically active component allicin and its derivative (Mikaili *et al.,* 2013). It has also been reported that these bioactive constituents are responsible for the antiviral, antibacterial, anti-fungal, and anti-protozoa activities of *A. sativum*. According to Amagase (2006), garlic has also been used to prevent different kinds of cancer including breast cancer, bladder cancer, colon cancer, stomach cancer, prostate cancer, rectal cancer, and lung cancer, and that it could be useful in the treatment of Cardiovascular disease including Antilipemic, antihypertensive, anti-atherosclerotic, an enlarged prostate, diabetes, osteoarthritis, cold and flu, and so on. It is also effective for building the immune system, preventing tick bites, preventing and treating bacterial and fungal infections.

Azadirachta indica is a member of the Meliaceae family of mahogany usually called neem or Indian lilac (USDA, 2020). It is typically grown in tropical and semi-tropical regions. The Siddha and Ayurvedic practitioners believed that Neem plant has anthelmintic, antifungal, antidiabetic, antibacterial, contraceptive, and sedative properties (Agrawal, 2013, Ismail *et al.,* 2020). The plant is believed to be the main constituent of Unani, Ayurvedic, and Siddha medicine in the treatment of skin diseases (Tamilnadu, 2012). Short-term use of neem is safe in adults but long-term use may harm the kidneys or liver in small children. Neem oil has been documented to enhance healthy hair, detoxify the blood, ameliorate liver function, and balance blood sugar levels (Tamilnadu, 2012).

Tetrapleura tetraptera belonging to Fabaceae family is also called Aidan in Yoruba, Uhio in the Igbo language of Nigeria is a species of flowering plants in the pea family which is native to Western Africa (Margaret, 1988); it is also called Prekese or Soup Perfume in the Twi language of Ghana (Osie-Tutu *et al.,* 2010). In Tropical African traditional medicine, its fruit is frequently used for the treatment and management of some of human diseases such as hypertension, diabetes mellitus, hypertension, epilepsy, arthritis, and other inflammatory conditions, schistosomiasis, asthma, postpartum (after delivery) recovery, as immune system booster (Ojewole and Adewumi, 2004). The pod has been reported to contain polyphenol, flavonoid, tannins, and alkaloids which are antioxidants that protect the body from free radicals and oxidative damages responsible for aging.

Khaya grandifoliola belonging to the Meliaceae family is popularly called Oganwo in Yoruba native of Nigeria, and also called Benin Mahogany, African mahogany; Senegal mahogany is a tall woody tree, a medicinal plant endemic to Nigeria (Hutchinson and Dalziel, 1978). It is also found in Benin, the Democratic Republic of the Congo, Ivory Coast, Ghana, Guinea, Sudan, Togo, and Uganda. It is threatened by habitat loss. Traditionally, it has been reported to have been used in the form of concoction for the treatment of convulsion, cough, stomach ache, fever, threatened abortion, rheumatism, dermatomycosis, and malaria fever in Nigeria (Odugbemi *et al.,* 2007; Uroko *et al.,* 2020).

Heliotropium indicum known in English as Indian heliotrope is also called Agogo Igun in Yoruba native of Nigeria; it is an annual plant considered as a weed by

farmers, but as a valuable medicinal plant by traditional medicine practitioners. The plant is native to Asia. In Natore district, Bangladesh, a folk medicinal practitioner used the root for blood purification and to treat infection (Akhter *et al.*, 2016). The sap is applied to gumboil, to clean ulcers, and to cure eye infections in Nigeria and Ghana. It is also used to treat warts, inflames, and tumors. Throughout tropical Africa, it is used as an analgesic to ease rheumatic pain, as a diuretic and to treat numerous skin problems including yaws, urticaria, scabies, ulcers, eczema, impetigo. A decoction of the whole plant is used to treat thrush, diarrhea, diabetes, venereal diseases, and frequent excretion of urine (Burkill, 1935).

Opuntia dillenii belonging to the Cactaceae family of the genus Opuntia grows in dry and desert environments to a height of about 1 to 1.8 meters. It is a great medicinal herb and a shrub. The plant has suggested that the fruit may be useful as a medication for gonorrhea, whooping cough, and constipation, as well as controlling the bile secretion, spasmodic cough, and expectoration, while the leaves of the plant have been reported to have been used as a medication for wound and inflammation as well as a treatment for ophthalmic disorders (Raj, 2015; Kirtikar, 2006). Among the reported diverse pharmacological activities of this plant, anti-oxidant, anti-inflammatory, anti-tumor, neuroprotective, hepatoprotective, hypotensive, and immuno-modulation are the basis of the application of this plant in the preservation and treatment of some chronic diseases. Scientific studies on *Opuntia dillenii* can help better understand its pharmacological mechanism of action to elucidate its traditional uses and to identify its potential new therapeutic applications.

Capsicum frutescens is a member of the Solanaceae family with five domesticated species: *C. annuum, C. baccatum, C. chinense, C. frutescens*, and *C. pubescen* (Moscone *et al.*, 2007). They are normally very small and pungent. The fruit usually grows pale yellow and matures to bright red, but can also be other colors. This plant has been reported to have used in traditional medicine for the treatment of rheumatoid arthritis, osteoarthritis, digestion problems, and conditions of the heart and blood vessels. Pepper is one of the most important plants that have been used as medicine for a long time in different countries and civilizations. In old civilizations, it was used by the Mayas for treating asthma, coughs, and sore and by the Aztecs to relieve toothaches (Bosland, 1996). Dietary antioxidants have protective role against many diseases such as cancer, diabetics, cardiovascular, and anemia. Vitamins E, C, and β-carotene are important as protective antioxidants, and peppers are rich in vitamin C and E as well as carotenoids and xanthophylls (Perucka and Materska, 2007; Mateos *et al.*, 2013).

Turmeric is a medicinal plant of *Curcuma longa* which belongs to the *Zingiberaceae* family. It is popularly referred to as Atale or Ajo among the Yoruba speaking parts of Nigeria (Priyadarini, 2014; Oladele *et al.*, 2020a). Turmeric is a perennial plant. It is grouped among the rhizomatous and herbaceous plants. The rhizomes of *Curcuma longa* plants are gathered each year either for propagation in the next season or for consumption. The rhizome of *C. longa*, Linn has been reported to have many therapeutic activities such as anti-inflammatory, anti-diabetic, hepatoprotective, hypolipidemic, anti-diarrhoeal, anti-asthmatic, and anti-cancerous drug (Sastry, 2005; Sharma, 2006; Chunekar, 2010; Pandey, 2002; Oladele *et al.*, 2020a).

Honey is a sweet, viscous food substance made by honey bees and some related insects (Crane, 1990). Bees produce honey from the sugary secretions of plants (floral nectar) or secretions of other insects (such as honeydew), by regurgitation, enzymatic activity, and water evaporation. Bees store honey in wax structures called honeycombs (Crane, 1990). The variety of honey produced by honey bees (the genus Apis) is the best-known, due to its worldwide commercial production and human consumption (Al-kafaween *et al.*, 2020). Honey is collected from wild bee colonies, or hives of domesticated bees, a practice known as beekeeping or apiculture. Honey gets its sweetness from the monosaccharides: fructose and glucose, and has about the same relative sweetness as sucrose (table sugar) (NHB, 2012). The antimicrobial activity of honey against microorganisms such as bacteria, viruses, fungi, and protozoa has been reported in many scientific literatures (Carter *et al.*, 2016).

6. Conclusion

COVID-19 is a highly infectious and severe acute respiratory disorder caused by a pathogenic virus known as severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). Its clinical manifestations include fever, fatigue, cough, shortness of breath, and other complications. The mode of actions of SARS-CoV-2 includes hyper-inflammation characterized by a fulminant and fatal hyper-cytokinaemia with multi-organ failure; immunosuppression; reduction of ACE2 to enhance pulmonary vascular permeability and damage the alveoli and activated by ORF3a, ORF3b, and ORF7a via JNK pathway which induces lung damage. These mechanisms of action of the virus can be mitigated by combined therapy of the medicinal herbs based on their pharmacological activities. Furthermore, plant materials and natural products have been very effective in the treatment of symptoms related to COVID-19. Experimental research is needed to prove the efficacy of these medicinal plants and their product against COVID-19. As a recommendation, since COVID-19 is a multifactorial clinical disorder with co-morbidities, we strongly recommend the use of combined therapy with two or more herbs with specific therapeutic actions being administered to combat the key players in the pathogenesis of the disease.

Authors contribution

Conceptualization: OJO; Data curation: OJO, AEIO, OMO, OTO, OBD, ABM; Funding acquisition: OJO, AEIO, OMO; Investigation OJO, AEIO, OMO, OTO, OBD, ABM; Project administration: OJO, AEIO; Resources; OJO, AEIO, OMO, OTO, OBD, ABM; Supervision; OJO, AEIO, OMO; Roles/Writing - original draft: OJO, AEIO, OMO, OTO, OBD, ABM.

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Morphology and Distribution of *Empoasca decipiens* Paoli and *Asymmetrasca decedens* (Paoli) (Hemiptera: Cicadellidae), in Jordan

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Abstract

A survey was carried out from May 2018 to November 2019 to collect the green leafhoppers from different ecosystems in Jordan using a battery-operated vacuum device or a sweeping net from 27 localities in Jordan. The collected specimens were identified as *Empoasca decipiens* Paoli or *Asymmetrasca decedens* (Paoli). Both species were found widely distributed and overlap in their range. Accurate species identification depended only on the microscopic examination of male genitalia. Data on the distribution, collecting dates and morphology of these two species were given including color images of adults and male genitalia.

Keywords: Leafhoppers, survey, male genitalia, Typhlocybinae, Empoascini.

1. Introduction

The genus *Empoasca* Walsh, 1862 includes more than 643 species distributed worldwide while genus *Asymmetrasca* includes 17 species restricted to the Palearctic and Oriental regions (http://dmitriev.speciesfile.org, Liu *et al*., 2014). The green leafhoppers, *Empoasca decipiens* and *A. decedens* are widely distributed in the Middle East, North Africa, central Asia and most of Europe (Raupach *et al*., 2002). Both species are considered pests on economically important agricultural crops, including grape, tomato, potato, corn and other host plants (Raupach *et al*., 2002; Atakan, 2011; Emam *et al*., 2020). However, misidentifications of the two species caused confusion over their identity due to high morphological similarity to each other and closely related species (Poos, 1932; Karimzadeh and Dworakowska, 2011; Aguin-Pombo, 2014; Qin *et al*., 2015). For example, *E. punjabensis* has often been misidentified as *E. decipiens* (Karimzadeh and Dworakowska 2011). Al-Asady (2002) illustrated the morphology of male and female adults and the male genitalia of *A. decedens*.

The objectives of this study were to survey, identify the species of the green leafhoppers and contribute to their morphology to facilitate their identification.

2. Materials and Methods

Specimens were collected from 27 localities from various habitats in the Jordan including the highlands, Jordan Valley and desert areas from May 2018 to November 2019. In addition, specimens collected previously and preserved at the University of Jordan Insect Museum (UJIM) were examined. Locality names, their coordinates, host plants, collecting dates, number of males, number of unidentified females and the total number of specimens are given in Table 1. Coordinates were taken by Global Position System. Leafhoppers were sampled mainly by a battery-operated vacuum device (InsectaZooka, BioQuip products, Rancho Dominguez, CA) and occasionally by a sweeping net. Collected specimens were preserved in alcohol or pinned on cards and housed at the University of Jordan Insect Museum. Male genitalia of all collected specimens were removed and soaked in hot 10% KOH for 3-8 min, washed in distilled water, and then transferred to glycerin for further dissection and identification. Specimens were identified using the key of Le Quesne and Payne (1981) and the interactive key at (http://dmitriev.speciesfile.org), which included relevant literature about Jordan or other adjacent areas. Color photographs were taken for adult insects by Canon EOS 40 attached to Leica M165 C Stereomicroscope provided with Dome LED illumination. Male genitalia were photographed using the same camera mounted on a Leica DMLS binocular microscope. Body length for adults was measured from the tip of the head to the tip of the wings.

3. Results

A total of 368 specimens of leafhoppers were collected from 27 different locations on 28 host plants including grasses, fruit trees, vegetables, ornamentals and wild plants. Sampling sites, their coordinates, collecting dates, male and unidentified female numbers of both species are given in Table 1. Distribution of both species is shown in Figure 1. The examination of all male genitalia (Plates 1, 2 and 3) from each sample revealed that they belonged to

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either *Empoasca decipiens* and/or *Asymmetrasca decedens*. Morphological differences between *E. decipiens* and *A. decedens* male genitalia were shown in Table 2.

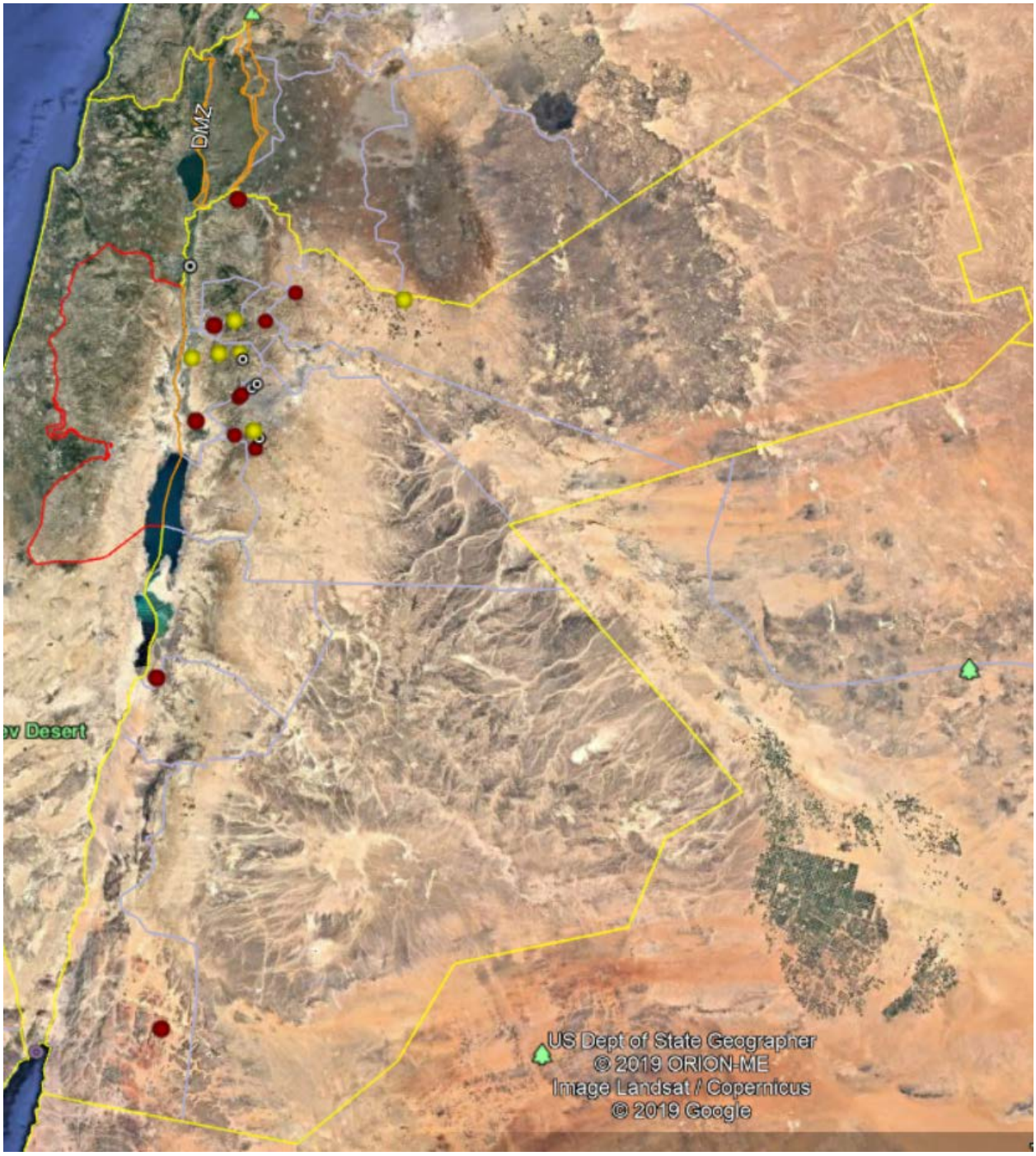


Figure 1. Map of Jordan showing the distribution of *E. decipiens* (Red) and *A. decedens* (yellow). The Black and white circle shows areas of overlapping populations. Source: Google Earth (US Dept. of State Geographer, accessed March 2020).

Table 1. Collecting localities, dates, hosts, number of males and total numbers of *E. decipiens* and *A. decedens*.

Locality	Coordinates	Host plant Common name, scientific name (family)	Collecting date	No. of *E. decipiens* male specimens	No. of *A. decedens* male specimens	No. of undetermined Female specimens	Total No. of specimens
Ash Shūnah ash Shamālīyah	N32° 28.15 E 35° 35.44	Citrus *Citrus* spp. (Rutaceae)	19/12/2017	4	2	8	14
Al Jubayhah, UJ* Campus	N32°00.695 E35°52.380	Field bindweed *Convolvulus arvensis* L. (Convolvulaceae)	30/5/2018	5	3	13	21
Al Jubayhah, UJ Campus	N32°00.695 E35°52.380	Rosemary *Salvia rosmarinus* Spenn. (Lamiaceae)	30/5/2018	2	-	6	8
Al Jubayhah, UJ Campus	N32°00.695 E35°52.380	Bermuda grass *Cynodondactylon* (L.) Pers. (Poaceae)	3/6/2018	-	1	1	2
Al Jubayhah, UJ Campus	N32°00.695 E35°52.380	Rosemary	3/6/2018	3	-	3	6
Al Jubayhah, UJ Campus	N32°00.695 E35°52.380	Johnson grass *Sorghum halepense* (L.) Pers. (Poaceae)	3/6/2018	3	-	4	7
Al Yadodah	N31°49.499 E35°54.465	Field bindweed *Convolvulus arvensis* L. and Mint *Mentha* sp. (Lamiaceae)	22/7/2018	3	-	4	7
Al Jubayhah, UJ* Campus	N32°00.695 E35°52.380	Citrus	1/8/2018	-	3	1	4
Ammān	N31°98.832 E35°83.039	Olives *Olea europaea* L. (Oleaceae)	6/8/2018	1	-	3	4
Ammān	N31°51.061 E35°52.888	Almond *Prunus dulcis* (Mill.) D. A. Webb (Rosaceae)	9/8/2018	-	1	-	1
Al Jubayhah, UJ Campus	N32°00.695 E35°52.380	Rose *Rosa* sp. (Rosaceae)	12/8/2018	6	4	15	25
Al Jubayhah, UJ Campus	N32°00.695 E35°52.380	Grape vine *Vitis vinifera* L. (Vitaceae) with *Convolvulus arvensis* beneath	12/8/2018	12	-	16	28
Al Jubayhah, UJ Campus	N32°00.695 E35°52.380	Rose	14/8/2018	1	-	4	5
Shafa Badran	N32°02.753 E35°89.996	False yellowhead *Inula graveolens* L. (Asteroideae)	14/8/2018	15	6	51	72
Al Yadodah	N31°49.499 E35°54.465	Silverleaf nightshade *Solanum elaeagnifolium* Cav. (Solanaceae)	19/8/2018	6	7	31	44
'Ayn ‘Aqrabah	N32°43.00 E35°48.00	Pomegranate *Punica granatum* L. Lythraceae	31/10/2018	3	-	3	6
Slaihi	N32°07.149 E35°49.886	Nettle *Urtica dioica* L. (Urticaceae)	20/1/2019	-	7	1	8
Dayr 'Alla, Damya	N32°07.149 E35°49.886	Potato *Solanum tuberosum* L. (Solanaceae)	26/1/2019	-	2	1	3
Tal ar Rummān	N32°07.149 E35°49.886	Brambles *Rubus canescens* DC. (Rosaceae)	30/1/2019	3	-	2	5

UJ Farm, Ghawr Kabid	N32°05.071 E35°35.706	Alfalfa *Medicago sativa* L. (Fabaceae)	13/2/2019	3	-	2	5
Dead Sea- Mādabā Road (Panorama)	N31°39.688 E35°34.964	Saltwort *Seidlitzia rosmarinus* Bunge ex Boiss. (Amaranthaceae)	20/2/2019	-	-	1	1
Dead Sea Hotels area	N31°44.340 E35°35.794	Purslane-leaved aizoon *Aizoon canariense* L. (Aizoaceae)	20/2/2019	-	-	4	4
Ghawr Fīfah	N30°55.640 E35°27.555	Toothbrush tree *Salvadora persica L.* (Salvadoraceae)	20/2/2019	2	-	1	3
Al Rawda	N31°50.001 E35°47.775	Thorny burnet *Sarcopoterium spinosum* L. (Rosaceae)	25/2/2019	2	-	2	4
UJ Farm, Ghawr Kabid	N32°05.071 E35°35.706	*Sorghum halepense* (L.) Pers. (Poaceae)	13/3/2019	2	-	3	5
UJ Farm, Ghawr Kabid	N32°05.071 E35°35.706	*Hopbush Dodonaea viscosa* Jacq. (Sapindaceae)	31/3/2019	-	-	3	3
Ash Shūnah al Janūbiyah	N31°53.215 E35°37.418	Grape vine	4/4/2019	3	-	7	10
Wādī Ramm	N29°36.809 E35°29.262	Devil's thorn *Rumex spinosus* L. (Polygonaceae)	12/4/2019	1	-	-	1
Aqaba	N30°07.833 E35°24.459	Ephedra *Ephedra* sp. (Ephedraceae)	3/5/2019	-	-	9	9
Ajlūn	N32°15.736 E35°47.506	Olives	8/5/2019	-	3	8	11
Rajeb	N32°14.601 E35°42.013	Oriental plane *Platanus orientalis* L. (Platanaceae)	8/5/2019	2	-	2	4
Slaihi	N32°08.573 E35°48.884	Thorny burnet	12/5/2019	-	1	-	1
Al Mafraq	N32°20.323 E36°32.521	Grape vine	19/5/2019	-	2	-	2
Umm al ‘Amad	N31°47.079 E35°53.414	Olives	29/5/2019	1	-	2	3
Jerash	N32°15.558 E35°55.758	Olives	11/6/2019	1	-	-	1
Al Jubayhah, UJ Campus	N32°00.695 E35°52.380	Rosemary	14/6/2019	-	1	-	1
Al Yadodah	N31°49.499 E35°54.465	Eggplant *Solanum melongena* L. (Solanaceae)	24/6/2019	-	3	7	10
Al Yadodah	N31°49.499 E35°54.465	Bermuda grass	24/6/2019	3	-	5	9
Wādī Bayodah	N32°08.326 E35°43.376	Oak *Quercus* sp. Fagaceae	11/7/2019	-	2	2	4
Wādī as Sīr	N31°58.487 E35°48.851	Oleander *Nerium oleander* L. (Apocynaceae)	20/9/2019	1	-	1	2
Al Mafraq	N32°21.934 E36°47.036	Bermuda grass	16/11/2019	1	-	4	5

*UJ: University of Jordan.

Table 2. Comparison between morphological characteristics of *E. decipiens* and *A. decedens* male genitalia.

Genitalia Structure	*E. decipiens*	*A. decedens*
Anal styli	Strongly curved, narrows gradually to a sharp point (Plate 2. A)	Slightly curved, end rounded not sharp (Plate 2. B)
Aedeagus	Symmetric, simple without a projection (Plate 2. A)	Asymmetric, with L-shape projection situated laterally and slightly beneath the apex. (Plate 2. C)
Paramere	Slightly curved in lateral view, distal part slightly curved tapered (Plate 2. D)	Almost straight in lateral view, distal part strongly curved (Plate 2. E)
Subgenital plate	Distal part slightly curved and narrow (Plate 2. F)	Distal part almost straight and truncated (Plate 2. G)
Pygofer appendage	Tapers to the middle part, then widens, and tapers again at the tip (Plate 3. A)	Tapers from the base gradually to a sharp tip (Plate 3. B)

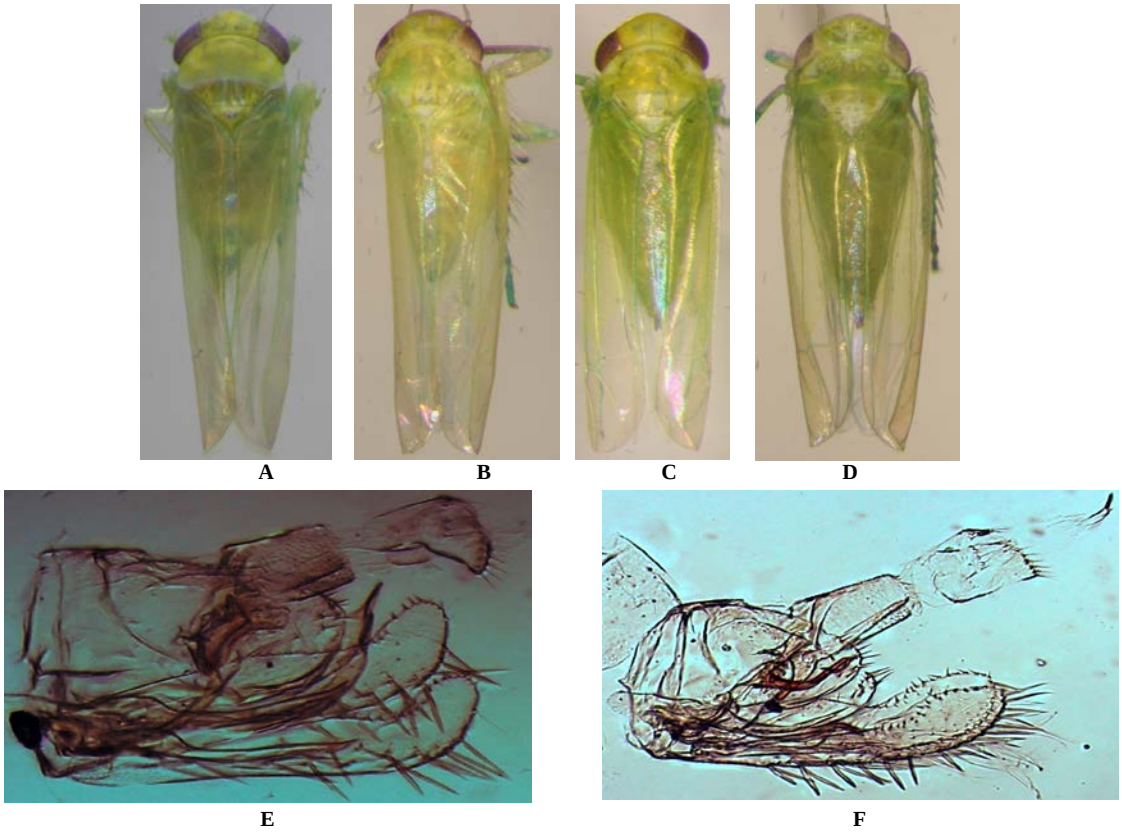


Plate 1. (A) Male of *E. decipiens*, dorsal view. (B) Male of *A. decedens*, dorsal view. (C) Female of *E. decipiens*, dorsal view. (D) Female of *A. decedens*, dorsal view. (E) Pygofer of *E. decipiens*, lateral view. (F) Pygofer of *A. decedens*, lateral view. Total body length: (A, C) = 3.3 to 4.1 mm, Total body length: (B, D) = 3.1 to 3.9 mm

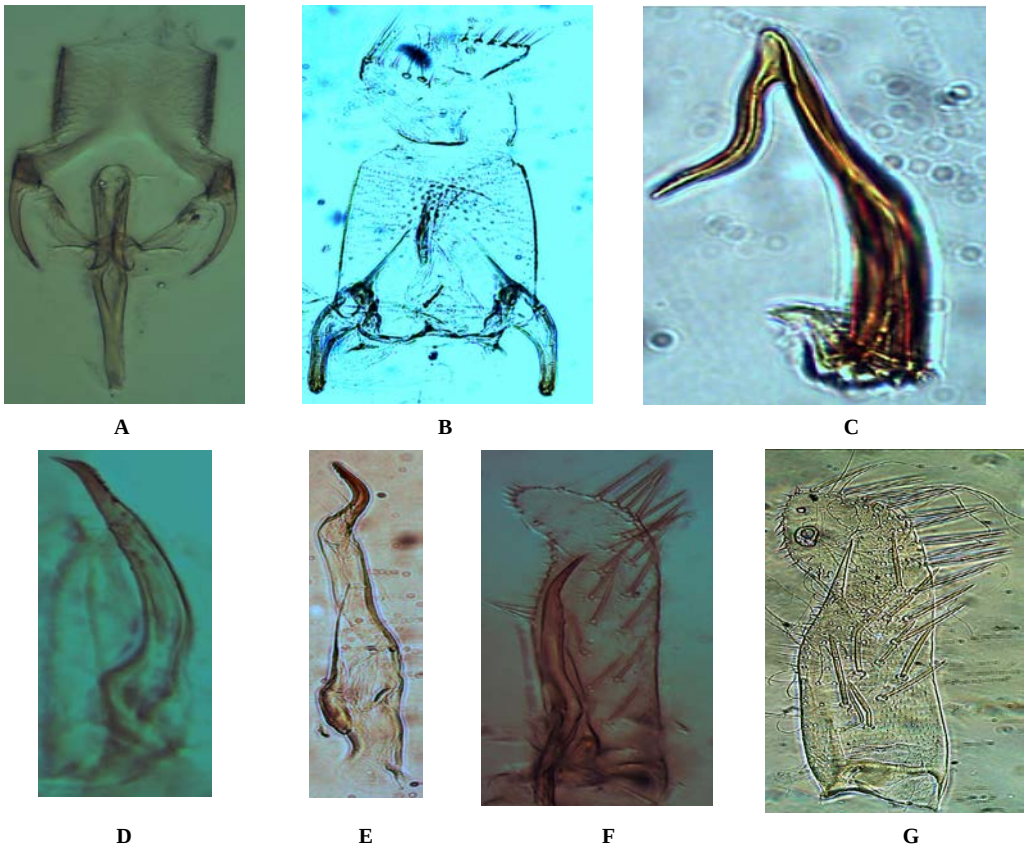
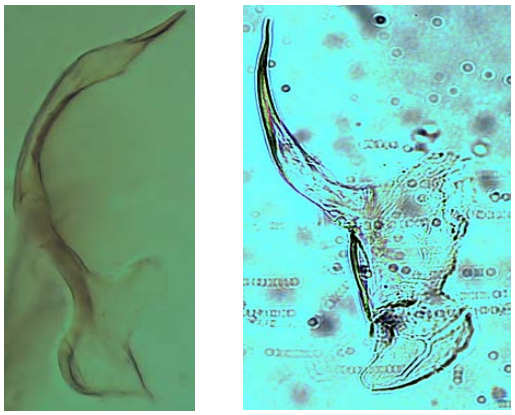


Plate 2. (A) Anal styli and Aedeagus of *E. decipiens* (B) Anal tube and styli of *A. decedens* (C) Aedeagus of *A. decedens* (D) Paramere of *E. decipiens* (E) Paramere of *A. decedens* (F) subgenital plate of *E. decipiens* (G) subgenital plate of *A. decedens.*



A B

Plate 3. (A) Pygofer appendage of *E. decipiens* (B) Pygofer appendage of *A. decedens.*

4. Discussion

Obtained data revealed that *E. decipiens* and *A. decedens* are widely distributed in Jordan and can be found on grasses, shrubs, ornamentals, fruit trees and vegetables (Table 1). *E. decipiens* was collected from pomegranate, brambles, alfalfa, toothbrush tree, devil's thorn, oriental plane and oleander. While *A. decedens* was collected from Almond, nettle, potato, eggplant and oak. However, either species could be found eventually on hosts of the other species since both are known to be highly polyphagous (Atakan, 2011; El-Wakeil *et al*., 2015; Emam *et al*., 2020).

The number of collected specimens of both species was relatively low; this could be attributed to the application of pesticides. Interestingly, in the arid nature of southern Jordan desert, *E. decipiens* was recorded form Wādī Ramm Reserve on *Rumex spinosus*. Moreover, it was found in Ghawr Fīfah on *Salvadora persica*, which faces habitat degradation due to intensive agriculture. In most cases, *E. decipiens* and *A. decedens* were found associated together on many hosts in different localities (Table 1).

The morphological similarity between the two species was high and both species can be misidentified by non-specialists. The two species were similar in size and color including the whitish patterns on the vertex and pronotum (Plate 1. A-D). Their identification is mainly based on the examination of male genitalia only. *A. decedens* can be distinguished from *E. decipiens* by the asymmetric aedeagus with L-shape projection situated laterally and slightly beneath the apex (Plate 2. C), unlike the simple aedeagus in *E. decipiens* (Plate 2. A). In lateral view, the distal part of *A. decedens* subgenital plate is almost straight and truncated (Plate 2. G), while in *E. decipiens* is narrow and curved (Plate 2. F). In *E. decipiens*, the tapered tips of the pygofer appendages are curved upward extending pygofer apex in lateral view (Plate 1. E) and (Plate 3. A). The ventral view of the anal stylus in *A. decedens* is slightly curved with thick rounded end (Plate 2. B), but in *E. decipiens* it is evenly curved with sharp end (Plate 2. A).

In general, the identification of cicadomoprphan species is difficult because of their homogeneity and lack of comprehensive identification keys and taxonomic literature (Dietrich, 2009). An increasing number of studies are using molecular markers as powerful tool for the identification of taxonomically difficult species in attempts to understand phylogenetic relationships and population structure (Demichelis *et al*., 2010 and Emam *et al*., 2020). Loukas and Drosopoulos (1992) distinguished *E. decipiens* from *A. decedens* populations in six different hosts using allozymes and male morphological examination. Such molecular techniques could be applied to female populations such as the ones collected from Aqaba, the Dead Sea and Ghawr Kbed.

Several studies confirmed *E. decipiens* and *A. decedens* as a potential vector of phytoplasma on different crops (Alhudaib, 2009; Alhudaib *et al*., 2009; Dakhil *et al*., 2011; Alsaleh *et al*., 2014). In Lebanon, it has been recorded as a potential vector of almond witches 'broom phytoplasma (AlmWB) (Dakhil *et al*., 2011). Galetto *et al* (2011) reported *E. decipiens* as a potential vector of *Chrysanthemum* yellows phytoplasma (CYP, "Ca. Phytoplasma asteris", 16SrI-B) in Italy.

5. Conclusion

E. decipiens and *A. decedens* are widely distributed in Jordan, collected from a wide host range, found almost all the year around which increasing their potential role in plant disease transmission. They could be accurately identified and differentiated by the examination of male genitalia. Further sampling in locations not included in this study can contribute to a detailed distributional range of both species in Jordan.

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Assessment of Testicular Histomorphometric Parameters and Reticular Fibres Density on Testicular Tissue of Diabetic Wistar Rat Placed on *Auricularia Polytricha*

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ABSTRACT

Role of *Auricularia polytricha* on diabetes – induced male reproductive dysfunction is not well understood. Local Nigeria men use this macro fungi to manage sexual dysfunction without an appropriate scientific investigations. The objectives of this study was therefore to investigate the effect of *A. polytricha* on histomorphometric parameters and reticular fibre density in testes of diabetic rat. Thirty (30) adult male Wistar rats was grouped into six designated A to F with 5 rats in each group. Group A (Normal Control) were treated with normal saline, Group B (administered with 65mg/kg.bw of streptozotocin) served as diabetic control. Groups C, D, E and F where placed on 250mg/kg.bw, 500mg/kg.bw, 1000mg/kg.bw of *A. polytricha* and 68mg/kg.bw of streptozotocin (STZ) respectively after inducing diabetics. Histomorphometric Measurements (seminiferous tubular diameter and germinal epithelial height) were done using ocular micrometre. Reticulum stain kits were used to demonstrate for reticular fibre. Results revealed that diabetic control group had remarkable increase in density of reticular fibre when compared to normal control and had tubular diameter (167.22 $\pm$ 0.10µm) that were significantly lower ($p<0.05$) when compared with normal control (310.81 $\pm$ 0.45 µm). Germinal epithelial height in Group B was also significantly ($p<0.05$) lower when compared to normal control. Reticular fibre expression was decreased in Groups C, D, E, and F in a dose dependent manner. However, tubular diameter and epithelial height were significantly higher ($p<0.05$) in groups placed on graded doses of *A. polytricha* when compared to diabetic control. Thickening of testicular interstitium as a result of reticular fibres density, and decrease in tubular diameter and epithelial height induced by diabetes were regulated and reversed following administration of graded doses of *A. polytricha.* Reversal in the reticular fibres expression may be due to powerful antioxidant potentials of *A. polytricha.* This study illustrates that supplementation with *A. polytricha* may confer protection for diabetes-related infertility.

Keywords: *Auricularia polytricha,* Diabetes, Histomorphometric parameters, Reticular fibers, seminiferous tubules, testes

1. Introduction

Diabetes will affect reproductive outcome of a fraction of diabetic men before and during their child-bearing ages (Saleh et al 2002). According to reports, approximately 50% of diabetic men will suffer from reproductive dysfunction within five years of the diagnosis (Saumya et al 2016). Consequently, it has become imperative to provide both preventive and curative measures to infertility in diabetic men. Furthermore, diabetes-related infertility has been ravaging a great population of young people with its attendant complications especially in rural communities where regular or periodic medical check is absent due to poverty and ignorance (Saleh et al., 2002).

Diabetes is thought to affect male reproductive function at multiple levels due to its effect on the endocrine control of spermatogenesis as well as penile erection and sperm quality. Studies in diabetic animal model have demonstrated an impairment of sperm quality and fecundity, structural defect and significantly lower motility of sperm cells (Baccetti et al., 2002), lower ability to penetrate zona free hamster eggs, detrimental effects on endocrine control of spermatogenesis and impairment of erection and ejaculation and progressive genotoxicity (Baker et al., 2004).

The gradual shift to herbal therapy with its attendant increasing acceptance, even among the elites confirm the claim that herbal remedies can provide cure for several diseases, including infertility in men (Killian et al., 2007). Many plant and animal products have been tested for possible fertility regulatory properties (Anthony, 2006). However, local Nigerian men have been reported to use *Auricularia polytricha* (wood ear mushroom) growing in farm lands and dead woods to manage sexual dysfunction with little or no research to confirm its efficacy

Studies in a diabetic animal model have demonstrated an impairment of sperm quality and fecundity, structural defect and significantly lower motility of sperm cells, lower ability to penetrate zona free hamster eggs, detrimental effects on endocrine control of spermatogenesis and impairment of erection and ejaculation, and progressive genotoxicity (Baccetti et al 2002). Also an increase in reticular fibre density around

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the seminiferous tubules threaten the spermatogenesis and may lead to infertility (Bhatia et al., 2010)

A correlation between histo-morphometric parameters and reticular fibre expression can therefore be useful in assessing histopathological alterations in testes (Mallidis et al., 2011).

In this study, we evaluate the ameliorative effects of *A. polytricha* on histo-morphometric parameters and reticular fibre expression on testicular tissue of streptozotocin (STZ)-Induced diabetic rat model treated with graded doses of *A. Polytricha.*

2. Materials and Methods

2.1. Preparation of Extract

Auricularia Polytricha was obtained from Etomi central market located in Etung Local Government Area of Cross River State and authenticated at the Department of Biological sciences, University of Nigeria. The mushroom was dried at room temperature, powdered and subjected to crude extraction using the ethanol modified method. 200g of *A. polytricha* was soaked in 1000ml of ethanol, labelled, covered and allowed to sit for 72 hours, after which a clean filter paper (Watman No 1) was used to filter extracts. The filtrate was evaporated to dryness at 40^oC in a vacuum using a rotatory evaporator. The extract was weighed and stored at 4^0C.

2.2. Experimental Animals

This study carried out in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals. Thirty (30) adult male Wistar rats with average weight of 150g were used for this research. The rats were kept in clean cages and divided into six groups designated A, B, C, D, E, and F with five rats in each group. The rats were allowed to acclimatize for two weeks in animal house, Department of Anatomy, Faculty of Medicine, University of Nigeria, Enugu Campus and allowed unrestricted access to commercially available chow (livestock feed) and water.

2.3. Experimental Design

Table 1. Experimental animals was divided into six (6) groups as follows:

GROUP	DESIGNATION	NUMBER OF ANIMALS	TREATMENT	DOSE
A	Normal control	5	Distilled water	3 mls
B	Diabetic control	5	Streptozotocin (STZ)	65mg/kg.bw
C	STZ + AP (Low Dose)	5	STZ + *A. polytricha*	250mg/kg.bw
D	STZ + AP (Mid Dose)	5	STZ + *A. polytricha*	500mg/kg.bw
E	STZ + AP (High Dose)	5	STZ + *A. polytricha*	1000mg/kg.bw
F	STZ + Metformin	5	STZ + Metformin	40mg/kg.bw

2.4. Induction of Hyperglycaemia

After fasting for twelve hours, diabetes was induced by administering streptozotocin (STZ) intra-peritoneally, reconstituted in 0.5M Sodium citrate and administered at a dose of 65mg/kg.bw (Ugochukwu and Babady 2003).

2.5. Confirmation of Diabetes

Diabetes was confirmed three days after administration of STZ using Accu-Check glucometer (Roche diagnostic, Germany) with blood samples obtained from tails of Wistar rats. Blood glucose levels (mmol/l) was checked before and after induction to ascertain hyperglycaemic state.

2.6. Administration of Extract

Experimental animals were kept for two weeks after inducing diabetes to allow for sustained hyperglycaemia after which *A. polytricha* extract administration commenced by oral gastric intubation and lasted for 21 days. The experimental protocol was maintained for a total of 35 days.

2.7. Termination of Experiment and Collection of Samples for Analysis

At termination, the animals were sacrificed with the testes removed and blotted with filter paper. Both right and left testes were weighed together and then suspended in Bouins fluid for fixation, preparatory to histological processing.

2.8. Histomorphometric Measurement

Histomorphometric Measurements were carried out using ocular micrometre after calibration on a light microscope. The stage micrometer was 1 mm long with 100 divisions so each division of the stage micrometer was one one-hundredth of a mm (0.01mm or 10 um). The eyepiece micrometer was divided into 100 units. 30 divisions of the reticle (eyepiece micrometer) corresponded to 200 micrometers. Measurements of seminiferous diameter and germinal epithelial heights were taken and recorded.

2.9. Reticular fibre examination

Reticulum stain kits - Modified Gomori's ((Sigma-Aldrich Products, Germany) was used in this research as a staining protocol for reticular fibre and basement membrane examination.

Silver nitrate solution was added in small amounts to the methenamine solution, mixing after each addition. A white precipitate formed which re-dissolved on shaking. Stock solution used was clear. The stock solution was filtered into a brown bottle and stored at 4^oC.

Procedure:

Slides were deparaffinised and then put into distilled water and then oxidize in 0.5% periodic acid solution for 15 minutes at room temperature. Slides were rinsed again 3 times in distilled water then incubated in methenamine silver working solution for 1 hour at 60^oC. Slides brearing tissue sections were then rinsed in hot distilled water followed by water at room temperature. Sectioned were toned in gold chloride and rinsed again in distilled water, treated with sodium thiosulfate for 2 minutes, rinsed and counterstained in nuclear fast red. Sections were finally dehydrated in xylene and mounted on a microscope.

Results showed black for basement membrane and reticular fibres with pink or green nuclear background.

2.10. Statistical Analysis

Quantitative data from this research was recoded and tabularised. Statistical significance of the differences between the groups was determined using one-way analysis of variance (ANOVA) done with SPSS, version 20 statistical analysis program. P<0.05 was considered as significant.

3. Results

3.1. Reticular fibre and basement membrane examination

Methenamine Silver (Gomori PAM) staining protocol for reticular fibre and basement membrane examination on testicular tissue was negative in group A (Normal Control) as there was no reticular fibre expressed in the basement membrane (Figure 1). However, observation showed positive result in group B (Diabetic control) which revealed the black colour reticular fibre seen round the seminiferous tubules (Figure 2). Density of reticular fibre was also increased in the diabetic control animals. Reticular fibre density in the section of testis in diabetic animals placed on 250mg/kgbw of and 500mg/kgbw of *A. polytricha* (Group C and D) was not remarkably decreased (Figure 3 and 4). Even though the result was positive, reticular fibre density was not as remarkable as that expressed in the diabetic control animals. Section of testis in groups E and F showed negative result with methenamine silver stain (Figure 5 and 6).

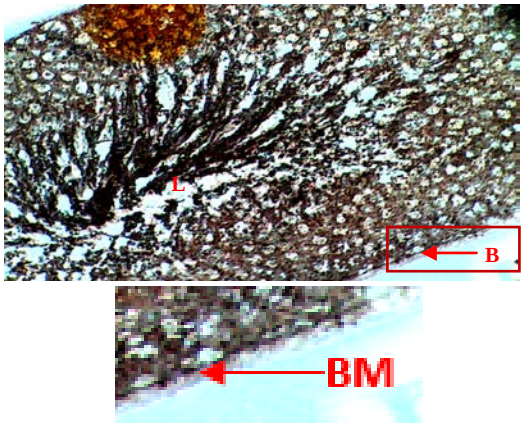

Figure 1. GROUP A (Normal Control) X400

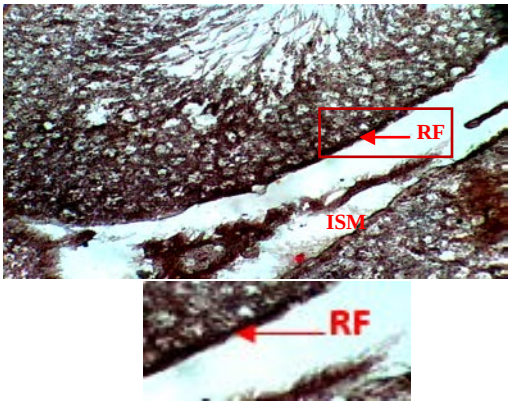

Figure 2.GROUP B (Diabetic Control) - X400

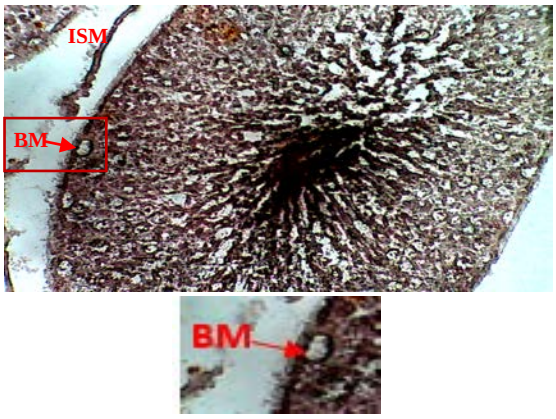

Figure 3. GROUP C - STZ + AP (250 mg/kg.bw) X400

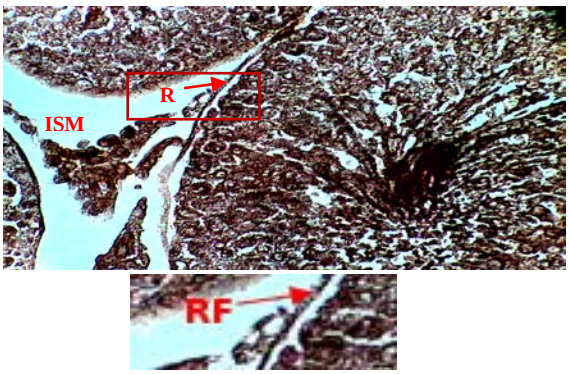

Figure 4. GROUP D, STZ + AP (500mg/kg.bw) X400

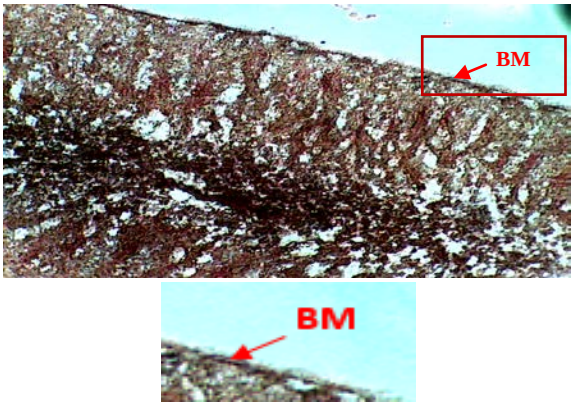

Figure 5. GROUP E - STZ + AP (1000mg/kg.bw) X400

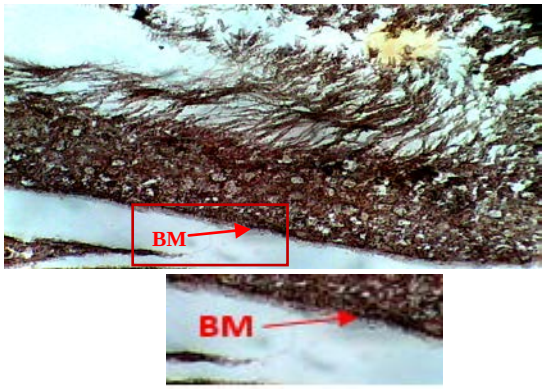

Figure 6 . GROUP F (STZ + Metformin) X400
Section of testis showing decreased formation of reticular fibres

KEY : L – Lumen , ISM – Testicular Interstitium , BM – Basement Membrane , RF – Recticular Fibres.

3.2. Histo-Morphometric Parameters

3.2.1. Tubular Diameter

Seminiferous tubular diameter and germinal epithelial height were the histomorphometric parameters considered in this study. 167.22 ± 0.10µm recorded as diameter of seminiferous tubules in Group B (Diabetic Control) was significantly decreased when compared to 310.81 ± 0.45µm in Group A (Normal Control). Section of testis in group C did not show any significant difference ($p<0.05$) in terms of tubular diameter when compared to Group B. However, among the diabetes induced groups, animals placed on 500 and 1000mg/kgbw of *A. polytricha* showed significantly (at $p<0.05$) increased tubular diameter (210.22 ± 0.45 and 249.10 ± 0.33 respectively) when compared to low dose group (Figure 7)

3.2.2. Germinal epithelial heights

Germinal epithelial heights were least in groups B (Diabetic control) and group C (Diabetic animals that received 250mg/kgbw of *A.* polytricha), measuring 10.11 ± 0.55 µm and 10.50 ± 0.72µm respectively, and were considered significantly decreased ($p<0.05$) when compared to epithelial height in Normal control - Group A (19.03 ± 0.23µm). Group E (1000mg/kgbw of *A.* polytricha) recorded the highest value in epithelial height (15.00 ± 0.34µm) among the diabetic groups and these values were significantly ($p<0.05$) higher when compared to Diabetic control - Groups B and C. However, interventions in Group D (500mg/kgbw of *A.* polytricha) and F (standard antidiabetic drug – metformin) showed a significant increase in the height of germinal epithelium when compared to the diabetic control (Figure 8).

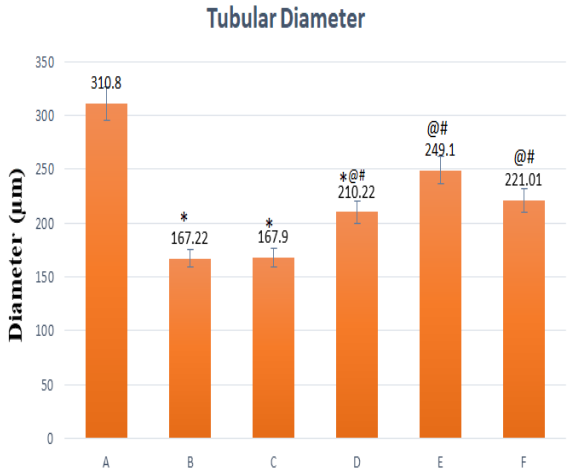

Figure 7: Comparison of Tubular Diameter in the different experimental groups.

Values are expressed in Mean ± SEM. N = 5.

***** = Values are significantly decreased when compared to Normal Control at $p<0.05$. **@** = Values are significantly increased when compared to Diabetic Control at $p<0.05$.# = Values are significantly increased when compared to Low Dose group at $p<0.01$.

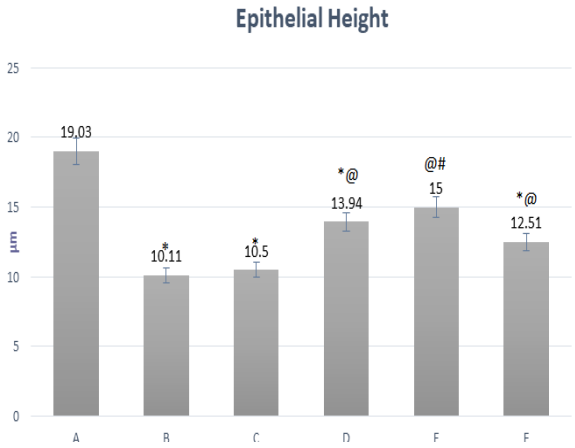

Figure 8: Comparison of Germinal Epithelial Height in the different experimental groups.

Values are expressed in Mean ± SEM. N = 5.

***** = Values are significantly decreased when compared to Normal Control at $p<0.05$. **@** = Values are significantly increased when compared to Diabetic Control at $p<0.05$.# = Values are significantly increased when compared to Low Dose group at $p<0.01$.

4. DISCUSSION

The effect of *A. polytricha* on testicular histomorphometric parameters and reticular fibre density in diabetic rat was investigated in this study. It was observed that seminiferous diameter and germinal epithelial heights which were significantly lower in diabetic control rats when compared to normal control group increased significantly following treatment with *A. polytricha*. Thickening of reticular fibre in diabetic animals was reversed following *A. polytricha* administration. From the aforementioned result, it is evident that diabetes have caused increase in the density of reticular fibre in the basement membranes of seminiferous tubules. The underlying mechanism may be connected with generation of reactive oxygen specie (ROS) in sustained hyperglycaemic condition capable of initiating a cascade of degenerative process that may result in altered structure of basement membrane. Another possible mechanism could be associated with the effect of hyperglycaemia on somatic cells due to rapid destruction of cell membranes via lipid peroxidation. Andreyev *et al.*, (2005) reported that excessive generation of ROS in hyperglycaemic condition, without a corresponding balance in antioxidant production capacity may cause cellular damage.

Correlation between extracellular matrix expansion as a result of increased expression in reticular fibres density and some histomorphometric parameters especially seminiferous tubular diameter and germinal epithelial height are considered significant variables in assessing testicular tissue damage and possible reversal. Increased reticular fibres density around the seminiferous in testicular tissues of diabetic control group as observed in this study indicates matrix expansion and as a consequence, smaller diameter of the seminiferous tubules and decreased germinal epithelial heights. This may imply that the higher the reticular fibres density, the wider the interstitial matrix and the lower the integrity of basement

membrane. Spermatogonia located at the basal compartment differentiates while moving to the adluminal compartment and a stable basement membrane is required for this cellular migration (Kilian et al., 2007). Increase in reticular fibre around the seminiferous tubules threatens the integrity of basement membrane and may affect spermatogenic process leading to infertility (Kilian et al., 2007; Baker et al 2004)

Decrease in density of reticular fibre and higher values of tubular diameter and epithelial heights following observed in diabetic animals following administration of *A. polytricha* may have been due to a great number of therapeutic, nutritive and biologically active components in *A. polytricha* that may have given protection against tissue degeneration.

Reactive oxygen species (ROS) which are increased by hyperglycaemia both in type 1 and type 2 diabetes, will cause oxidative stress (Beckman et al.,2001), which in turn plays a role in the development of complications in diabetes (Baynes et al., 1999). Chang et al., (2011) and (Mau et al., 2001) reported that *A. polytricha* is capable of inhibiting lipid peroxidation in body organs thereby restoring their weight and are known exogenous source of antioxidant because of the presence of bioactive β-D-glucan polysaccharides which are the major therapeutic and nutritional components found in cell walls of *A. polytricha.*

Findings from Chen et al., (2011) and Sun et al., (2010) reveal that uronic acid has been fractionized from fruiting body of *A. polytricha* and that the higher the uronic acid content of β-D-glucan polysaccharides, the more effective the antioxidant activity of the polysaccharide. Liao et al., (2014) and Chen et al., (2008) demonstrated that *A. polytricha* polysaccharides improved significantly, total antioxidant capacity and lipoprotein lipase activity in mice but was found to reduce melondialdehyde levels and arteriosclerosis index in rats and he attributed the strong antioxidant status to the phenolic compound in *A. polytricha*.

Photomicrographs of sections of testicular tissue in groups D, E and F placed on 500mg/kg.bw, 1000mg/kg.bw *A. polytricha* and STZ respectively revealed a reversal in reticular fibres expression and recorded significantly ($p<0.05$) higher tubular diameter and germinal epithelial heights when compared to the diabetic control. Some underlying mechanisms may have been responsible. Firstly, *A.* polytricha is a powerful exogenous source of antioxidants. Luo et al., 2009 reported that polysaccharides fractionated from *A. polythrica* formulation proof to have potential antioxidant effect against hydroxyl and superoxide radicals. This may have been responsible for inhibiting lipid peroxidation and improve on the microstructure of testicular tissues. Chen et al., 2011 reported similar findings. Secondly, the protein content in *Auricularia species* of mushroom was found to have significant amount of glutamic acid, lysine, alanine, glutamic acid, serine, and threonine and other essential free amino acids which may have provided the needed nutritional and therapeutic ingredients needed for repairs.

5. Conclusion

Findings from this study suggest that ethanolic extract of *A. polytricha* can reverse altered histomorphometric parameters and reticular fibre density in testicular tissue of diabetic rat model. This reversal was dose dependent. Clinical application of this research may suggest supplementation with *A. polytricha* on individuals with diabetes related infertility.

5. Ethical clearance

Ethical clearance was obtained from the Ethical Committee, Faculty of Basic Medical Sciences, University of Nigeria, Enugu Campus, Nigeria

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Competing Interest

All authors in this research are not by any means linked to any funding body and so there was a complete absence of external influence.

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Preliminary Studies on the fluctuation of the biomass of size-fractionated zooplankton in sea grass bed of Pulau Tinggi, Malaysia

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Abstract

Zooplanktons biomass was extensively studied in the sea grass bed of Pulau Tinggi, Malaysia for six months. In 2015, sampling months were April, June, October, whereas in 2016, April, June, August were the sampling months. A cone shaped plankton net was used with 0.30 m mouth, 1.00 m length and 100 µm mesh size. The fractionation of zooplankton size was carried out in to >2000 µm (large), 501-2000 µm (medium) and <500 µm (small). Zooplankton was classified as copepods, larvaceans, chaetognaths, cnidarians, ctenophores, decapods and polychaetes. Copepods were categorized as Calanoida, Poecilostomatoida, Cyclopoida and Harpacticoida but identified as a total of 54 species, 26 genera and 19 families. We conclude that among the biomass of 3 size fractions; medium (36%) was dominant followed by large and small (32% each) throughout the study period.

Keywords: Size-fractionated, sea grass bed, zooplankton, copepods.

1. Introduction

Zooplankton have key position in the aquatic environment, as they trophically link phytoplankton and higher order consumers. Zooplankton are consumers lying at the second trophic level (Manjare, 2015). Zooplankton play an important role in aquatic food chains and respond to a wide variety of disturbances, including nutrient loading (Dodson, 1992; Hossain *et al*., 2006; Begum *et al*., 2007; Paulose and Maheshwari, 2008). Zooplankton serve as food for benthic invertebrates and many other important, commercial fishes in their larval, juveniles and adult stages (Hossain *et al*., 2007; Duarte *et al.*, 2014). Many fish species use zooplankton as food during their larval stages, and some of them depend on zooplankton prey throughout their lives (Bates, 2007).

Menon and Pillai (2001) discussed the role of zooplankton as food for both juvenile and adult fishes. Zooplankton play a pivotal nutritional role both in captive fisheries and aquaculture (Imaobong, 2013). Rajagopal *et al.* (2010) suggested that zooplankton serve as a (a) bio-indicator and (b) tool for understanding the status of water pollution. Similarly, Ahangar *et al.* (2012) reported that zooplankton react strongly to environmental changes and can be used as an indicator of water health. According to Bhavimani. (2016), the zooplanktons are microscopic in size, ranging from micrometers to millimeters (Figure 1).

Sea grass habitats are essential in maintaining environmental and economic functions. Here, habitat is defined as a major ecological area occupied by the sea grass community (Ooi *et al*., 2011). Sea grasses trap sediment due to terrestrial runoff. It is considered as a nursery for many invertebrates and fishes. Sea grass beds are important ecosystems in tropical coastal waters for biological production and diversity. This complex ecosystem shows highest biodiversity for zooplankton (Metillo *et al*., 2018).

Metillo *et al.* (2018) studied the relationship of zooplankton community structure to environmental conditions in Pulau Tinggi, Malaysia. As there is paucity of information regarding biomass in size fractionated zooplankton there, therefore our research was carried out there to determine the zooplankton biomass in various size fractions.

Figure 1. Image of a zooplankton taken in the UTHM Laboratory with the help of a compound microscope (Olympus model SZX16)

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2. Materials and Methods

Sampling site

Johor is the southernmost state of Peninsular Malaysia and on the East coast as surrounded by the South China Sea (Mohamed *et al*., 2015). The coastline of Johor is ~436 km. The state government has identified ~72 islands in Johor state, 13 of them declared as Marine Parks, collectively known as the Sultan Iskandar Marine Park (SIMP).

Pulau Tinggi is included in one of the six main Marine Park Islands in Johor. Sampling for the zooplankton was done there at ten sea grass stations (Figure 2). The sampling site is a protected area; unapproved collection of living animals is restricted.

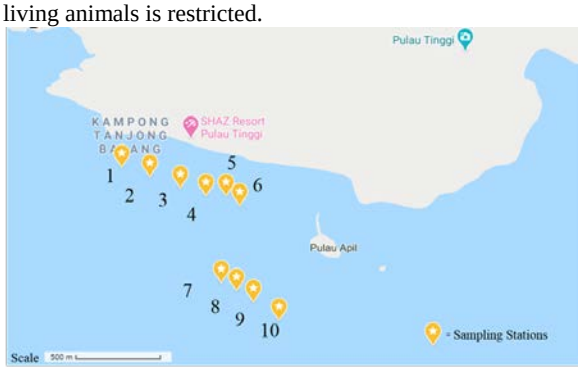

Figure 2. Map showing the station locations in Pulau Tinggi, Johor, Malaysia

Collection of Water Samples

Zooplankton sampling was conducted in the morning on each trip. Sampling was performed by using a plankton net with a mouth of 0.35 m, 1.00 m net length and 100µm mesh size. The plankton net was lowered to possible depth (9.4 m) for a while, then pulled to the surface where the filtered water was put in to a 250 ml bottle. Three aliquots of each water sample were collected from each station. Water samples were immediately preserved in 10% buffered formalin with a pH of 8.0-8.2.A total of 6 water samples were collected; April, June and October of 2015 and April, June and August of 2016 from the sea grass bed of Pulau Tinggi. Sampling was not carried out during rough weather, as caused by heavy rainfall and high tides. Physico-chemical parameters were measured *in situ*; dissolved oxygen (DO), temperature, pH, salinity, turbidity and conductivity with the help of a multiparameter meter (Hanna model HI 9829).

Water samples processing in the laboratory

The collected zooplankton samples were taken to the laboratory of University of Tun Hussein Onn Malaysia (UTHM) and further analyzed for density and biomass of 3 size fractions. In the laboratory, the water in each bottle was divided into two equal parts with a Folsom plankton splitter (Nakajima *et al.*, 2008), as shown in Figure 3. One part was used for identification and enumeration and the second part was used for biomass determination. The samples were examined with the help of a dissecting microscope. One sample was divided into three aliquots and zooplanktons were size fractionated into three fractions; > 2000 µm (large), 501-2000 µm (medium) and < 500 µm (small). The pH of the samples were checked for three consecutive days and maintained in the range of 8-8.2 in the laboratory. The pH of the preservative must be in this range to assure that the water samples are in good condition (Turner, 1976).

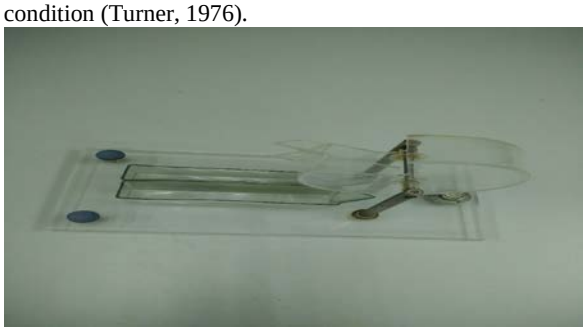

Figure 3. The Folsom plankton splitter used to divide the sample into two halve

Biomass determination

Biomass determination of zooplankton involved the ash-free dry mass method of Harris *et al*. (2000). For this purpose, ~100 ml distilled water was passed through a glass fiber filter (GFC) with the help of low pressure vacuum pump. Then each filter paper weight was noted and the filter papers were dried for two hours in an oven at 60 ºC. Then a desiccator was used to cool the filter papers before reweighing. The filtrates were transferred to a porcelain evaporating dish and combusted for two hours at 500°C inside the muffle furnace. The resultant ash was allowed to cool, then weighed with an electronic balance (Harris *et al*., 2000). The biomass of zooplankton was calculated using the following formula:

Ash-free dry mass (mg/ m^3) = A-B/V

A = Dried sample with filter in porcelain evaporating dish (mg)

B = Ash on filter in porcelain evaporating dish (mg)

V = Amount of filtered water (m^3)

Identification

Zooplankton were identified into seven taxonomic groups. Copepods were further identified up to species level following Conway *et al*. (2003), Boxshall and Halsey (2004) and Razouls *et al*. (2005, 2020). The identification of copepods was performed by examining the body shape, antenna segments and caudal rami structure (Maqbool *et al.*, 2015).

3. Results

The monthly zooplankton biomass ranged from 2.11± 0.45 to 7.47± 2.38 mg/m^3 during the present study. The physico-chemical parameters study showed that high temperature was recorded in April 2015, April 2016 and June 2016. The average water temperature for the sampling area ranged from 28.87 ± 0.99 to 31.50 ± 0.99 ˚C (Figure 4). Salinity ranged from 30.94 ±1.55 to 34.79±1.55 ppt in our study. The highest salinity value was observed in June 2016. Two salinity peaks, 33.00 ±1.55 and 34.79 ±1.55 ppt were recorded in June 2015 and June 2016 respectively. In other months the salinity was comparatively lower. Dissolved oxygen was in the range of 6.0 ± 0.30 to 6.9 ± 0.30 mg/L during the sampling months. The highest oxygen concentration was observed in June 2016 (Figure 4). The highest biomass was recorded in June 2016, a month in which the highest temperature, salinity and dissolved oxygen were recorded from the study site (Figure 4). Meanwhile, the lowest biomass was

observed in August 2016, a month in which the lowest temperature and oxygen was recorded at the study site. Based on total biomass, the medium size fractioned zooplankton was somewhat dominant over the large and small fractioned zooplankton (Figure 5). The medium sized fractioned was represented by 35.54%, of the total fractioned biomass followed by the large sized fraction (32.42%) and small sized fraction (32.03%) as shown in Table 1. In the year 2015, the samples of April and June 2015 showed the dominance of the small sized fraction followed by large and medium size fractions. In the samples of October 2015, the large sized fraction was highest in abundance followed by the medium and small sized fractions. In the year 2016, all the three samples obtained in April, June and August yielded consistent results, as the medium sized fraction dominated in the biomass (Figure 6).

Zooplankton were identified as copepods, larvaceans, chaetognaths, cnidarians, ctenophores, decapods and polychaetes. Copepods were further classified as Calanoida, Poecilostomatoida, Cyclopoida and Harpacticoida. A total of 26 genera and 19 families of copepods were found in the samples of zooplankton collected from the sea grass bed of Pulau Tinggi.

Table 1. Average biomass and percentage of zooplankton size fractions during our study at the Pulau Tinggi sea grass bed. (N) number of fraction repetition, (SE) standard error

Fraction (µm)	Biomass ± SE	Percentage	N
<500	1.46 ± 0.77	32.03 %	6
501-2000	1.62 ± 1.05	35.54 %	6
>2000	1.47 ± 0.76	32.43 %	6
Total	4.55	100 %	18

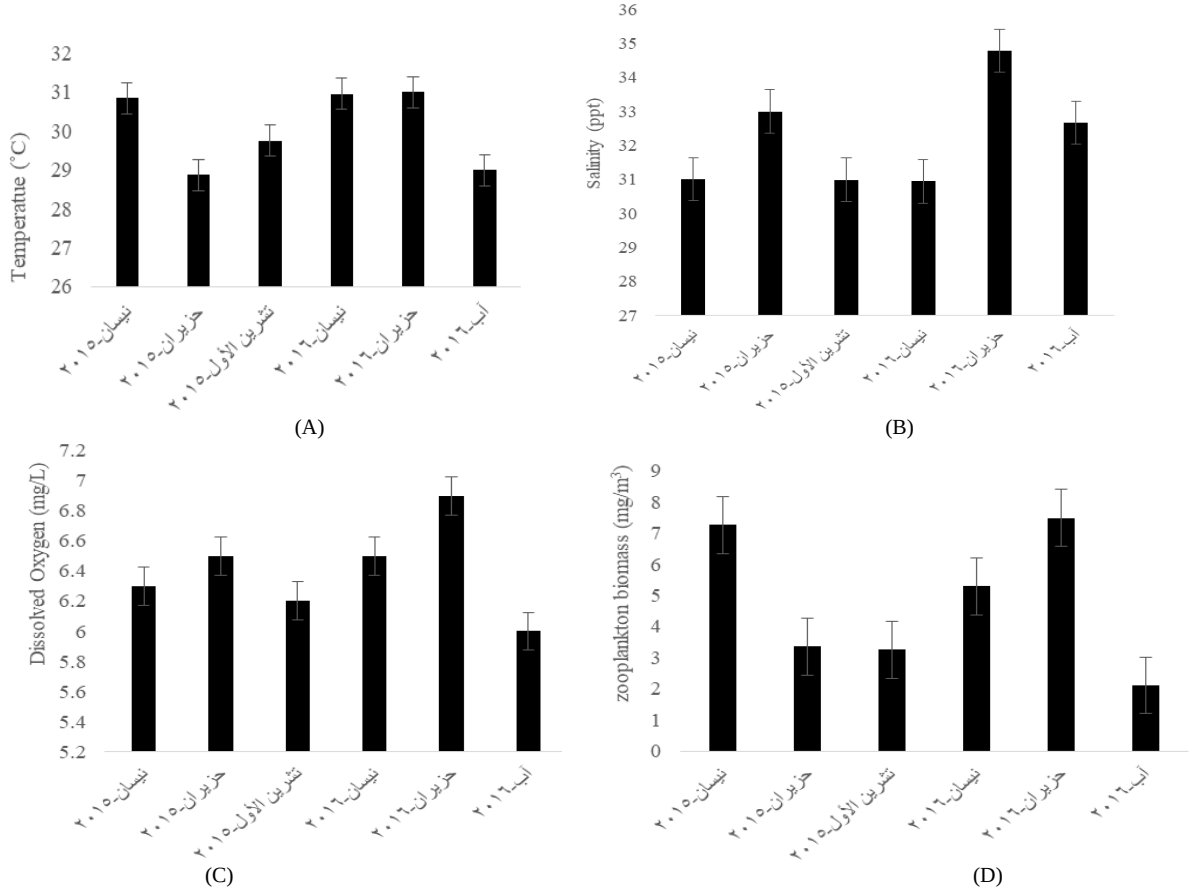

Figure 4. Fluctuations of physico-chemical parameters and zooplankton biomass (A= temperature; B= salinity; C= dissolved oxygen; D= zooplankton biomass)

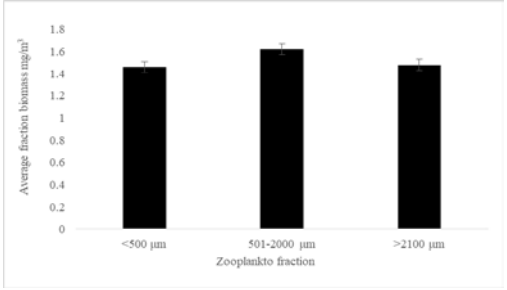

Figure 5. Average biomass (mg/m^3) of zooplankton size fraction sampled during our study at the Pulau Tinggi sea grass bed

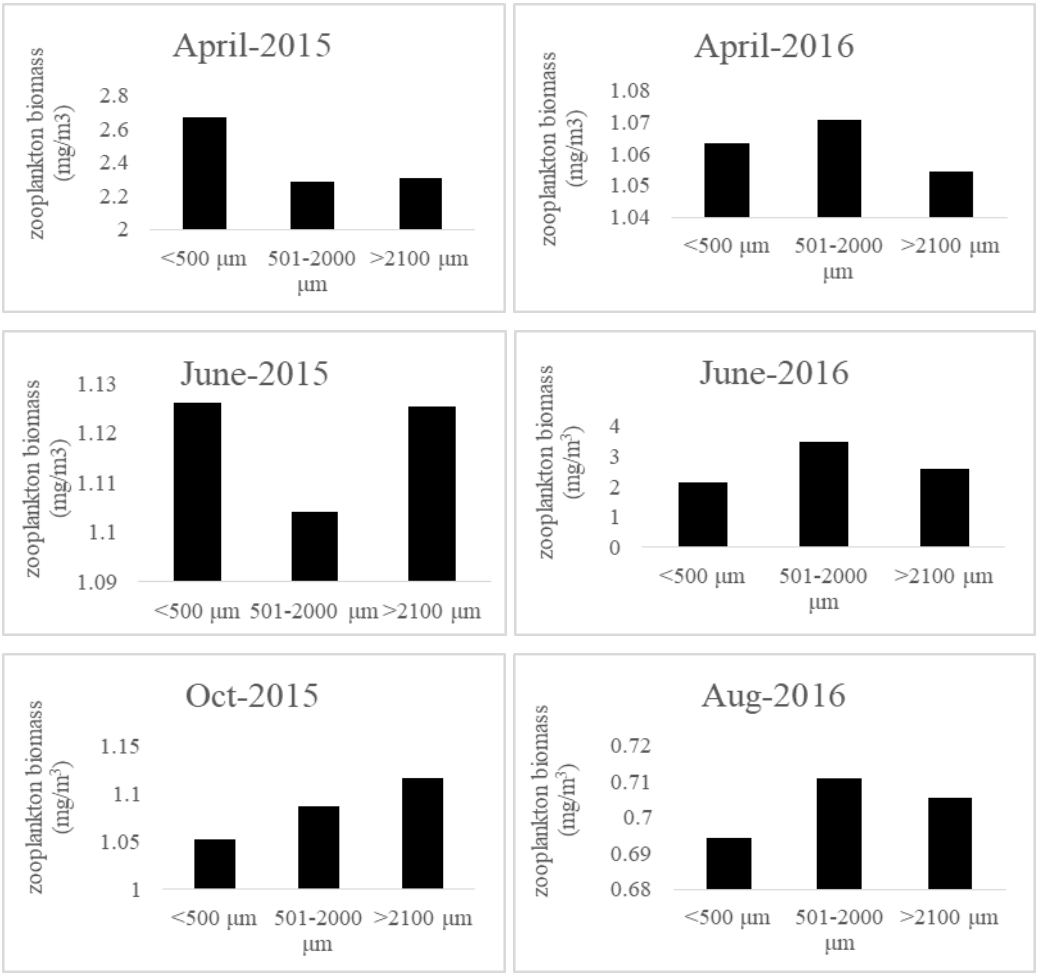

Figure 6. Fluctuation of the average biomass (mg/m^3) in zooplankton size fractions sampled at the Pulau Tinggi sea grass bed

4. Discussion

In the present study, higher biomass was observed in those months when the temperature, salinity, and DO were higher in the study area. This may be related to the fact as reported earlier that the zooplankton biomass is regulated by physico-chemical parameters and tide in the estuary (Fatema *et al*., 2016). Similarly, in one study there was a statistically significant difference in zooplankton biomass across seasons, being highest in autumn 2009 but in spring for other years (Kurt and Polat, 2015). Furthermore, the study was done in the Straits of Malacca where zooplankton biomass was higher in coastal than offshore areas via river runoff and mangrove forests (Rezai *et al*., 2003). But the biomass recorded in our study (Figures 4-6) was lower than for the tropical mangroves estuary in the Straits of Malacca (Balqis *et al*., 2016). This may be related to the fact that their study (Balqis *et al*., 2016) was conducted for one full year while in our present study we have covered three months in a year. In our results, the biomass of medium sized zooplankton (501-2000 µm) yielded the highest biomass in most months of our study. Abundance of different sized zooplankton has been reported in different habitats, for example, Nakajima *et al*. (2008) found that the 200-335 µm sized zooplankton were dominant yielding 74.3% of the total abundance in the coral-reef ecosystem of Redang Island. Another study in the coastal waters of the Gulf of Aqaba by Al-Najjar and El-Sherbiny (2008) reported that zooplankton with size fraction > 500 µm were dominant, yielding 67% to the total biomass. Zooplankton abundance, biomass, and size structure were studied in the coastal waters of the Northeastern Mediterranean Sea, where, small-sized zooplankton (200–500 µm) were dominant (Kurt and Polat, 2015). On the other hand, small copepods (100-335µm) were greatest in number in the sea grass beds of Pulau Tinggi (Metillo *et al*., 2018), where we found that biomass of medium size zooplankton (501-2000 µm) was highest in most months of our study.

5. Conclusion

Zooplankton was classified into seven taxonomic groups; copepods, larvaceans, chaetognaths, cnidarians, ctenophores, decapods and polychaetes. The copepods were categorized into four orders; Calanoida, Poecilostomatoida, Cyclopoida and Harpacticoida. This included a total of 54 species 26 genera and 19 families of copepods. We conclude that the medium fraction was somewhat dominant in biomass for the whole study period, as compared to the large and small size fractions.

6. Conflicts of Interest

The authors declare that there is no conflict of interest in publishing the current paper.

Acknowledgments

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Jordan Journal of Biological Sciences **Short Communication 1**

Predicting Blood Lead Levels Using Endogenous Lead Levels in the Scalp Hair of Workers in Acid Battery Manufacturing Plant: A Pilot Study

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Abstract

Twelve hair samples were collected from a selected representative worker group working in a battery plant. The hair samples were analyzed for lead (Pb) levels using Atomic Absorption Spectrophotometry (AAS). Blood lead (BPb) levels were predicted from the results of hair samples lead levels following application of Clayton and Wooler (1983) model. The arithmetic mean for both endogenous Pb levels and predicted BPb levels were 8.95 ± 7.66 mg/g and 85.26 ± 22.57 µg/100 mL respectively. The pearson correlation coefficient between endogenous Pb levels and predicted BPb levels was (r = 0.88). The arithmetic mean BPb level was above the internationally reported acceptable level. Endogenous Pb level in scalp of hair samples is considered as a good specimen for predicting BPb level.

Keywords: Blood lead level, prediction, hair, battery plant, biomarker

1. Introduction

Lead (Pb) poisoning is considered a significant environmental disease (Wani et al., 2015). Its effects on the human body are deleterious, which appears in damaging the cardiovascular system, hematopoietic system, nervous system, kidneys, and reproductive system (Needleman et al., 1996).

Occupational exposure is believed to be the major cause of Pb poisoning in adults, as manifested in workers of battery plants (Qasim and Baloch, 2014). Approximately, 85% of the total global consumption of Pb is for Pb-acid batteries production (ILA, 2017). In many countries, acid battery manufacture exposure is almost unregulated, and monitoring of exposure rarely exists (Manhart et al., 2016). Therefore, Pb fumes and dust that are generated from such operations pose an exceptional health risk not only to the workers inside the factories but also to individuals living near these operations (Rodney, 1985).

Although BPb Level is considered the dominant biological marker used in workplace monitoring and clinical assessment regarding removal from exposure (Qasim and Baloch, 2014), other biological samples like hair, breast milk, nails, and urine had usually been used for trace elements, including Pb, determination in human body become very important. Hair has many advantages for its easy and painlessly collection, easy transport, storage, and low cost (Dombovári and Papp, 1998). Unlike blood, hair is less vulnerable to reactive homeostatic mechanisms that affect trace element levels (NBO, 2020).

The validity of using hair sample as alternative to blood sample in determination of Pb levels in workers occupationally exposed should be examined. In Jordan, there are many acid battery manufacturing plants which are considered as a potential major source of Pb emissions and pollution (Nsheiwat et al., 2010). The main aim of this study was to determine endogenous hair Pb level as a marker of lead exposure in workers of acid battery manufacturing plant located in Marka, Jordan. The obtained results were applied to evaluate the validity of using endogenous Pb levels in predicting BPb levels in same workers.

2. Materials and methods

2.1. Chemicals

Lead free concentrated nitric acid (HNO_3, 69-71%) of spectroscopic grade was obtained from Gainland chemical factory, UK. Acetone (99.6%) of spectroscopic grade was purchased from Fisher scientific. Lead standard solution 1000 ppm was obtained from BDH laboratory supplies, UK. Deionized water from analytical reagent grade water purification system (Millipore) was used.

2.2. Sample collection and preparation

All laboratory equipment used for the sample collection and sample treatment were cleaned and soaked with 10% nitric acid overnight, rinsed using deionized water, then dried with acetone.

Hair samples were collected from twelve different healthy adult Jordanian males working in acid battery plant that is located in Marka (north eastern part of Amman). Hair was cut from different zones of the scalp (Feisal et

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al., 2019). Each hair sample was cut into pieces as small as possible and mixed to allow representative sub-sampling. 200 mg of each hair sample was accurately weighed and stored in polyethylene bags at room temperature until analysis. Also, seven control hair samples were collected from seven different persons in a non-contaminated area.

2.3. Sample wash

200 mg of each hair sample was washed and agitated two times with 20 mL deionized water into 250 ml Erlenmeyer flask (Krueger and Duguay, 1989). Contact time of the distilled water with the hair sample was 20 min each time. The two volumes of deionized water wash were combined into separated labeled 50 ml volumetric flask and preserved for further analysis for determination of exogenous lead. Washed hair samples were dried at 110 ºC and kept for determination of endogenous lead.

2.4. Sample digestion for exogenous Pb determination

Each wash of hair sample, separated in 50 mL, was transferred into individual clean 100 mL beaker. After that, each beaker was heated to around 100 ^{o}C on a hot plate under a fume-hood until most of the water was evaporated. Then, 10 mL of concentrated nitric acid was added to each beaker and covered with a watch glass. Each beaker was heated to approximately 120 ^{o}C until most of the liquid was evaporated. Additional 5 mL of nitric acid were added to each beaker and digestion continued until the digests were clear and about 2-3 mL of acid remained. The digests were cooled for 1 hour, diluted to 50 mL with deionized water and kept for analysis by AAS.

2.5. Sample digestion for endogenous Pb determination

Each washed hair sample was transferred into individual clean 100 mL clean beaker. 10 mL of concentrated nitric acid was added to each beaker and covered with a watch glass. The beakers were heated to approximately 120 ^{o}C on the hot plate under a fume-hood until most of the liquid was evaporated. Another 5 ml of concentrated nitric acid was added to each beaker and digestion continued until the digests were clear and 2-3mL of acid remained. After that, the digest was cooled for 1 hour, diluted to 50 mL with deionized water, and kept for analysis by AAS.

2.6. Prediction of BPb levels in acid battery workers

Prediction of BPb levels for workers of the current study was performed using their endogenous hair Pb levels, by applying the model developed by Clayton and Wooler (1983).

2.7. Instrument working conditions

Atomic absorption spectrophotometer with flame atomization (SpectrAA. 250 plus Varian), (department of chemistry, faculty of science, the University of Jordan) was used (Al-Subeihi, 2002).

2.8. Quality Assurance and Control

Determination of Pb levels in hair samples was performed based on the analysis method validated by Al-Subeihi (2002). Reagent blank samples were digested similar to hair samples and were used to correct instrument readings. Analysis of blank samples revealed no detection for any trace of Pb. Accuracy and precision of the method were validated by six replicate measurements using spiked hair samples at concentrations of 5 ppm and 20 ppm (Table 1) (Al-Subeihi, 2002).

Table 1. Accuracy and precision results (Al-Subeihi, 2002)

Spiked Pb (µg/ mL)	*No. of samples	Measured Pb (µg/mL)	Accuracy (Relative error)	Precision (STDEV)
5	6	4.35	13	0.124
20	6	19.14	4.3	0.886

2.9. Data analysis

Since the size of measurements in this study was small ($n < 30$), the normality of the data had been assessed appropriately using Shapiro-Wilk test using SPSS. The one sample t-test (one tailed) was used to test whether the sample mean of the study group is significantly different from a reference value of non-contaminated group. Results were considered significantly different when $p < 0.05$.

3. Results

3.1. Determination of endogenous Pb in hair samples

The mean level of Pb in hair samples of workers was 8.95 ± 7.66 mg/g, and ranged from 0.26 to 22.63 mg/g (Table 2). On the other hand, The mean Pb level in hair samples obtained from persons living in a non-contaminated area was < 0.2 µg/g. The Shapiro-Wilk test result ($p = 0.217$) indicated that the sample came from a normal population. A one sample t-test (one tailed) between the reference value obtained for endogenous Pb levels in scalp hair of individuals of the non-contaminated area (< 0.2 µg/g) and the mean level of Pb of the battery workers indicated that there was a significant difference between the two groups ($p = 0.001$).

Table 2: Predicted BPb Levels

Sample no.	Endogenous Pb (mg/g)	Exogenous Pb (mg/g)	Predicted BPb levels (µg/100 mL)
1	0.70	32.63	57.16
2	22.63	0.12	109.04
3	9.32	5.57	95.80
4	2.71	0.90	77.36
5	8.56	0.19	94.53
6	4.65	5.81	85.42
7	0.26	0.91	42.38
8	0.56	8.36	53.83
9	13.33	10.34	101.14
10	21.23	8.80	108.09
11	12.60	9.47	100.30
12	10.82	0.314	98.03
Mean	**8.95**	**6.95**	**85.26**
STD	**7.66**	**9.02**	**22.57**

3.2. Prediction of BPb levels in acid battery workers

Clayton and Wooler (1983) measured BPb and hair Pb levels of 38 male workers at a Pb acid battery plant in Sydney (Clayton and Wooler, 1983). The correlation between BPb and hair Pb levels in the study of Clayton and Wooler was significant: $r = 0.76$. The data followed an exponential curve: $Hair\ Pb\ level = 15.2e^{0.067 * BPb\ level}$

where the BPb level is expressed as µg/100 mL and the hair Pb level is expressed as µg/g (Clayton and Wooler, 1983).

BPb levels in acid battery plant workers of this study were predicted by extrapolation, using Clayton and Wooler (1983), the results ranged from 42.37 to 109.04 µg/100 mL (mean of 85.26 ± 22.57 μg/100 mL). The result of Shapiro-Wilk test ($p = 0.06$) suggested that the sample came from a normally distributed population. According to the Occupational Safety and Health Administration (OSHA, 1991), BPb levels of 40 µg/100 mL or more suggest possible Pb toxicity. A one sample t-test (one tailed) indicated a significant difference between the reference value of OSHA and the mean value of workers of the acid battery plant ($p < 0.0005$).

3.3. Quality Assurance and Control

Analysis of blank samples revealed no detection for any trace of Pb. Accuracy and precision of the method were validated by sex replicate measurements using spiked hair samples at concentrations of 5 ppm and 20 ppm (Table 1). The results of the observed Pb concentration were in excellent agreement with the certified values and the method recoveries were 87 ± 2.9 % and 95.7 ± 4.6 %, respectively (Al-Subeihi, 2002).

4. Discussion

Pb intoxication is common among persons chronically exposed to high Pb levels. Occupational exposure is the main cause of Pb poisoning in adults, as in Pb miners, battery workers, smelters, plumbers, glass manufacturers, cosmetic workers, etc.

In Jordan, the main source of occupational Pb toxicity is acid battery manufacturing plant. This pilot study aimed at measuring endogenous hair Pb level as a marker of Pb exposure in workers of acid battery manufacturing plant located in Marka, Jordan. The obtained results were applied to evaluate the validity of using endogenous Pb levels in predicting BPb levels in the same workers.

The average of endogenous Pb level in hair samples of workers was 8.95 ± 7.66 mg/g, and ranged from 0.26 to 22.63 mg/g (Table 2). The variation in endogenous Pb levels might be justified in that some individuals worked for long periods in the battery plant than others, which revealed a long term exposure to Pb. On the other hand, Pb in hair samples obtained from persons living in a non-contaminated area was < 0.5 mg/g. The results obtained for endogenous Pb in hair revealed high deposition levels of Pb. It was concluded that occupational exposure to Pb is reflected by elevated levels of scalp hair Pb of exposed humans in polluted areas.

It was reported that Pb level in scalp hair of some workers of Pb battery plant was 2.3 mg/g (Noguchi et al., 2014), where the average Pb hair levels of the current study was 3.9 fold higher. In one study, the average Pb hair level was about 0.363 mg/g (Fergusson et al., 1981). It was noticeable that the present findings were around 25 fold more than that reported by Fergusson et al. Elevation of endogenous Pb levels in scalp hair may demonstrate long term exposure to Pb in the acid battery plant.

Washing procedure using de-ionized water was sufficient enough to remove the deposited Pb. This was inferred by noting the results summarized in Table 2. For example, analyzing the wash of scalp hair in samples (1, 6, and 8) to measure the exogenous Pb level revealed high Pb levels whereas the endogenous Pb levels were low. The variation in exogenous Pb levels as shown in table 2 might be justified in that size of work inside the battery plant differs from one day to another. Due to this difference, different amounts of Pb had been released to the atmosphere of the plant and cause different deposition of Pb on the outer surface of workers scalp hair.

BPb levels in acid battery plant workers of this study were predicted by extrapolation, using Clayton and Wooler (1983); the results ranged from 42.37 to 109.04 µg/100 mL (mean of 85.26 ± 22.57 μg/100 mL). Strong correlation is expected between endogenous Pb levels in scalp hair and the predicted BPb levels, and the correlation is r=.88. This correlation supported using Pb level in scalp hair to predict BPb level in exposed individuals. These predicted results were comparable with the results of workers involved in Pb acid battery plant in Bangladesh (78.70 μg/100 mL) (Ahmad et al., 2014). Also, a mean BPb level of 96.70 ± 27.90 μg/100 mL had been reported in a study performed in Tehran, Iran (Reihani and Niktab, 2005).

According to OSHA, BPb level should be below 40 μg/dL where levels more than 40 μg/100 mL, worker should be informed in writing and provided with medical examination. Moreover, workers having BPb level of 60 μg/100 mL for a single time must be removed from their job and could be placed to a job of lower exposure (OSHA, 2018). Workers with BPb level above 60 μg/100 mL commonly suffer from chronic nephropathy which may progress to kidney failure (Gidlow, 2007). All predicted BPb levels in the workers of the current study are greater than 40 μg/dL and around 75% were greater than 60 μg/100 mL while 66.7% are greater than 80 μg/100 mL (Table 2). Workers with BPb levels of 40 μg/100 mL or more suggest possible intoxication with Pb and willingness to show the symptoms of Pb poisoning (OSHA, 1991).

Qasim and Baloch (2014) reported 2 cases of occupational Pb poisoning in adult battery workers where BPb levels in case 1 and case 2 were 98.83 and 120.20 μg/100 mL, respectively. Both male patients had initial non-specific symptoms of intermittent abdominal pain, fatigue and headache for 6 - 8 years. Later on, they developed psychosis, slurred speech, tremors of hands and initially underwent treatment for Parkinsonism and Wilson's disease because of clinical misdiagnosis. Therefore, it could be concluded that the workers of the acid battery plant were chronically exposed to Pb and were at the risk of developing Pb toxicity.

5. Conclusion

Pb concentrations in hair wash and endogenous Pb levels in hair of battery plant workers were relatively high compared to control hair samples collected from seven different persons in a non-contaminated area. It could be concluded that workers of battery plant are at high risk for Pb pollution.

Strong correlation is expected between endogenous Pb levels in scalp hair and the predicted BPb levels and the correlation is $r = 0.88$. This correlation supported using Pb level in scalp hair to predict BPb level in exposed individuals.

6. Recommendation

Based on these results and conclusions, means to reduce exposure are highly recommended including reduction of air Pb concentrations. Moreover, monitoring of BPb levels among workers is strongly suggested. Also, educational programs of various workers group on the health and effects Pb should be held regularly.

Conflict of Interest

None to Declare.

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Jordan Journal of Biological Sciences **Short Communication 2**

Cyanoxanthomycin, a Bacterial Antimicrobial Compound Extracted from Thermophilic *Geobacillus* sp. Iso5

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Abstract

The simple and reliable method was developed for the extraction of a novel class of cyanoxanthomycin (3-(3,5,7,10-tetrahydroxy-1,1,9-trimethyl-2,6-dioxo-2,6-dihydro-1H-benzo[cd]pyren-4-yl) propanenitrile) type antibiotic from thermophilic *Geobacillus* sp. Iso5. The molecule was a typical cytoplasmic pigment, and can be readily identified (both in extraction and thin layer chromatography) by yellow-orange fluorescence under long UV/blue intensities. GC-MS analysis revealed that the molecular structure of the isolated pure extract was cyanoxanthomycin type antibiotic pigment composed of a benzopyrene with five fused rings in a planar delocalized structure. FT-IR spectra confirms the presence of hydroxyl, carbonyls, methyl and cyano group in the pigment. Furthermore, the *in-vitro* antibacterial cyanoxanthomycin was determined to have selective growth inhibition against various bacterial strains (Gram +ve/-ve). Further studies on various physiological and genetic level effects of this antimicrobial compound will provide the possibility for the future therapeutic and industrial applications.

Keywords: Thermophiles, *Geobacillus*, cyanoxanthomycin, fluorescence, pyrene

1. Introduction

Recent advancement in structural, molecular and genetics of thermophiles which were adopted to extreme temperature provided us with significant knowledge for their utilization in industrial and pharma biotechnology (McMullan et al. 2004; Stetter 1999). These thermophiles produce unique proteins, enzymes, cellular components and other biochemicals that function under conditions in which mesophilic counterparts cannot be sustained (Bull et al. 2000). Because of their unusual structural integrity of the molecules allow them to withstand harsh conditions typically associated with industrial processes, which has consequently resulted in them being used in several novel applications (Dufossé et al. 2005; Morozkina et al. 2010).

Thermophiles, as a result, is driven by the novel bioactive molecules found to be convenient in many applications. Despite, certain groups of hyperthermophilic bacteria can able to produce simple to more complex compounds in their existing environment. Especially, the production of fluorescent pigment is predominant in a member of the mesophilic group unless it is found less in thermophiles and hyperthermophilic bacteria. Although, a variety of plants, animals and microorganisms produce fluorescent pigments. Few bacterial groups produce blue, blue-green, yellow-orange and green-red coloured pigments, which are having different functions and chemical properties. On the other hand, the pigment productions on selective media are important taxonomic tools for the differentiation and classification of bacteria (Jordan 1899; Nazina et al. 2001). Most of the industrially viable fluorescent pigments used currently are extracted from plants and other mesophilic bacteria. It could be an advantage to produce natural fluorescent pigments from thermophilic bacteria due to their stability and other novel applicabilities.

The pentacyclic derived compounds from bacteria also exhibit fluorescent properties, which are functionally similar to other pentacyclic pigments from plants and other sources. Xanthomycin is one such pentacyclic compound containing cyano group which is absent or less predominant in hyperthermophiles. Some pigments are of cytoplasmic origin and produce characteristic yellow-orange colour in long UV radiation. The fluorescent pigment is less predominant in many bacterial genera, which exhibit the characteristic yellow-orange coloured fluorescent property. There are very few reports on cyano group containing crude/pure drug extracted from bacteria, especially *Bacillus* genus (Nair et al. 2019).

On the basis of its occurrence and distribution, there is no evidence of pentacyclic derived fluorescent pigment in thermophilic and hyperthermophilic groups especially in the members of bacterial group 5, *Geobacillus*. Here we have described a simple, rapid and novel method in isolation and characterization of yellow-orange fluorescent pigment from hyperthermophilic *Geobacillus* sp Iso5.

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2. Materials and Methods

2.1. Bacterial cultivation and culture media

Thermophilic aerobic *Geobacillus* sp. Iso-5 was isolated previously from alkaline thermal spring of Karnataka, India (Mahadevan 2012). To be precise, the strain was isolated from Bendruthirtha Hot water springs of Karnataka. The bacterium, *Geobacillus* sp. Iso5 is a gram-positive, non-motile, rod shaped with 1.3-1.5 µm in width and 3-6 µm in length. Its growth range was observed between 65–90 ºC, with an optimum at 70 ºC; the pH range for optimal growth is between 6 – 9. The mineral enrichment of LB cultured cells was aerobically transferred to SPYM media containing (w/v) 1 g of Starch; 0.5 g of Peptone; 0.2 g of yeast extract, 0.05 g of Sodium chloride, 1% vitamin mixture and 0.5% mineral salt solution. The medium was prepared pH 8.0 by adding either 1 N HCl or 1 N NaOH and sterilized. The culture was inoculated at 65 °C and kept for 48 hours under constant agitation. Tryptic Soy Agar (TSA) (HiMedia, Mumbai), Nutrient Agar (NA) (HiMedia, Mumbai) were also used to perform antimicrobial properties of the pigment extract.

2.2. Extraction of Fluorescent pigment

A desired seed culture of the bacterium was hygienically transferred to 2.5 liters of SPYM and incubated for 48 hours at 65 °C. The cells were then harvested by centrifugation at 10,000 × *g* in 4 °C for 25 min. The bacterial pellets were collected and washed with a minimum amount (~25 ml) of 50 mM Phosphate buffer (pH 7.0) and approximately twenty-five grams biomass was then freeze-dried. The freeze-dried sample was washed twice with of 80% ice-cold acetone and agitated for the two-minute cold condition. Finally, the agitated mixture was filtered using Whatman No 1 filter paper (Whatman, USA) under cold condition and air-dried. The filtrate was then extracted three times using under reflux for one hour by chloroform/methanol (2:1) (HiMedia, Mumbai) solvent mixture. After each subsequent reflux step, the extracts were combined and evaporated to dryness under room temperature.

2.3. Thin Layer Chromatography

The purity of the fluorescent pigment was analyzed by thin layer chromatography (TLC) using Silica Gel 120. The dried extract was dissolved in minimum amount of chloroform/methanol (2:1) and purity was determined under 0.5-mm silica gel sheets (Merck 60F-254) using chloroform-ethyl acetate (3:1) solvent system. For the extraction from silica gel, the preparative TLC was performed under thin Silica Gel plates (0.3 mm, Merck, Mumbai). The fluorescent compound was irradiated and visualized with a long-wavelength (340- 365 nm) UV lamp (Philips, USA) (Magyarosy et al. 2002). The fluorescent bands were scraped off from the plate, dissolved in minimum amount of ice-cold acetone, and filtered through Whatman No1 filter paper.

2.4. Analytical Methods

The GC-MS profile of the fluorescent pigment was determined by the VF-5ms 30 m x 0.25mm (ID) x 0.25µm quartz capillary column with helium as the carrier gas, injector temperature was 280 °C, split ratio 15:1.0 and sample size 1.0 µl for GC condition. The ionization mode was EI, electron bombarding energy was 70 eV, charging multiplier tube voltage at 500 V, scan range from m/z 40 to 650 at 3 scan/sec was used, and solvent delay at 3 min was maintained for mass determination. The infrared spectrum of the pigment was recorded on Nicolet Magna-IR 380 FTIR spectrometer (Thermo Electron, USA) spectrophotometer using KBr pellets at 32 average scans rate.

2.5. Antibacterial assay

The antibacterial property of the cyanoxanthomycin pigment was determined by agar well diffusion method using Muller-Hinton (MHA) agar plates. The concentration of 50µM (1 µM= 429µg/ml) was prepared by dissolving the appropriate amount of pure extract (cyanoxanthomycin) in sterile distilled water. The equal amount of standard ciproflaxin using distilled water was also prepared. The overnight grown individual bacterial strains were uniformly swabbed on to the surface of MHA plates by sterile cotton swabs. The cell concentration of *Bacillus subtilis* (MTCC 3053), *Escherichia coli* (MTCC 1698), *Pseudomonas aeruginoasa* (MTCC 6458), *Staphylococcus aureus* (MTCC 6908) and *Streptococcus* sp. (MTCC 9724) were 2.1×10^6, 2.6×10^6, 2.5×10^6, 2.9×10^6, and 3.1×10^6 cfu/mL, respectively (Mulla et al., 2016 a and b). Each 50µL of sample were aseptically loaded in well and the plates were incubated in an upright position for 1 day at 37 °C. The clear zone of inhibition around colonies indicated the antibacterial property of the pigment.

3. Results and Discussion

Extremophiles, isolated from the extreme ecological niches are well adapted to unfavourable environmental factors and have huge biotechnological potential (Dufossé et al. 2005; Fujiwara 2002; Singh and Gabani 2011). Especially, the biologically active substances synthesized by extremophiles, currently seeking to be important for their ability to survive and proliferate. These compounds are generally nonessential for growth of the organism and are synthesized with the aid of genes involved in intermediary metabolism. Among bioactive compounds of microbial origin have been characterized, a limited number of compounds is used for biomedical, agricultural and industrial applications. One such natural bioactive compound is fluorescent pigment with potent antibiotic properties.

3.1. Extraction and characterization of the Pigment

Herein, a simple procedure was developed for isolation of water-insoluble fluorescent pigment from *Geobacillus* sp. Iso5. It was characterized as yellow-orange colour producing pigment under long wavelength of UV intensity (Figure 1). The chloroform/methanol extraction (2:1) of pigment under reflux condition yielded a bright yellow-orange coloured spot on silica gel plate (data not shown). The purity of the extraction protocol was further analyzed by preparative thin layer chromatography, also revealed the presence of single yellow-orange colored spot (R_f value, 0.53) on the thin silica gel plates. Further fluorescent properties in different solvents such as acetone, dimethyl sulfoxide, methanol and pyridine also revealed the presence of same yellow-orange fluorescent colour.

The molecular mass of the pigment analyzed by using GC-MS revealed the presence of one major peak at *m/z* 429.14 with 90% intensity at 12 minutes (Figure 2). Additionally, from FT-IR, the CN stretching frequency is observed for the molecule between 2200-2300 cm^{-1}. From these results it was identified as cyanoxanthomycin of *Geobacillus* sp. Iso5 is a conjugated pigment. The production of fluorescent pigments by many bacterial groups was previously reported (Jordan 1899; Seleen and Stark 1943). Indeed, particularly abundant with the fluorescent *pseudomonads* adjacent to the atmosphere, spermosphere and rhizosphere in the soil (Elliott 1958; Huge 1964; Turfreijer et al. 1938). The *Pseudomonas fluorescens* produces yellow-green fluorescent pigment (YGFP) from brewery waste yeast with and without mineral salts in the medium (Silva and Almeida 2006). Elliott (1958) and Meyer (2000) described some of the properties of the water-soluble fluorescent pigment of the *Pseudomonads* called Pyoverdine. There are only a few reports available on the fluorescent pigment production from *Bacillus* sp. (Kolev 1991; Sneath 1986). Although, the influence of some natural and synthetic medium for the production of blue, blue-green, yellow-orange and green-red fluorescent coloured pigments from *B. fluorescens albus, B. fluorescens tennis, B. fluorescens mesentericus, B. fluorescens putridus. B. viridians and, B. fluorescens liquefaciens* was also reported (Flugge 1886; Jordan 1899; Meyer 1978; Seleen and Stark 1943). Further, Turfitt (1937) employed ordinary nutrient media for *B. pyocyaneus*, *B. fluorescens liquefaciens*, and *B. fluorescens non-liquefaciens* accompanied by the diffusion of a green or greenish blue fluorescence throughout the culture. However, there are only few *Bacillus* spp. produces yellow-green diffusible pigments were reported.

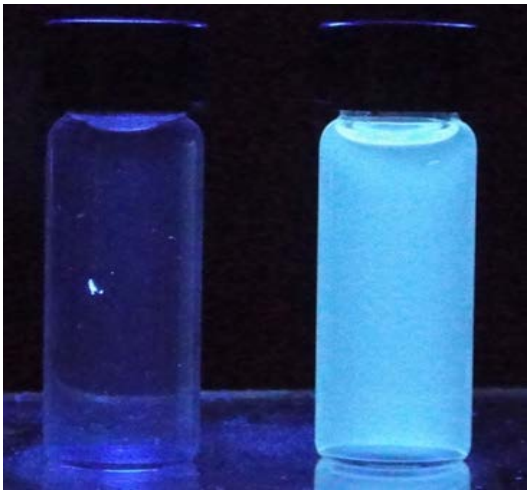

Figure 1 Flourescent property of chloroform/methanol without (A) and with extract (B). Dried extract was suspened in chloroform/methanol (1:1), and visualized by long-wavelength UV intensity (350 nm). "A" indicates the control system containing only solvent without extract did not show any folurescent property.

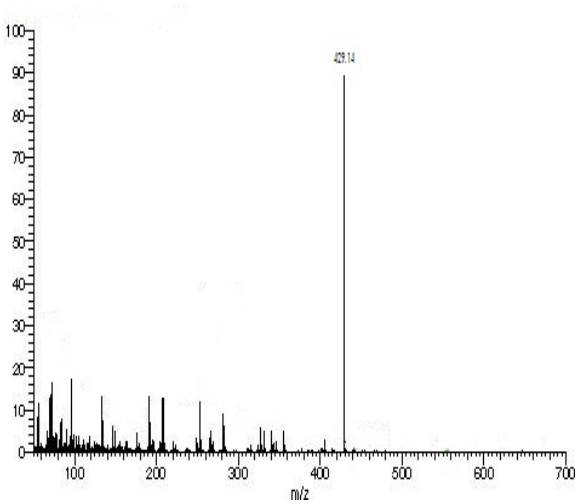

Figure 2 GCMS spectum of the purified chloroxanthomycine showing an single peak at 429.14 with 90% average intensity.

Description

Based on the spectral assignment, the cyanoxanthomycin (3-(3,5,7,10-tetrahydroxy-1,1,9-trimethyl-2,6-dioxo-2,6-dihydro-1H-benzo[cd]pyren-4-yl) propanenitrile) composed of a benzopyrene with five fused rings in a planar delocalized structure. There are four hydroxyl groups, two carbonyls, three methyl group and propanenitrile containing cyano group (Figure 3).

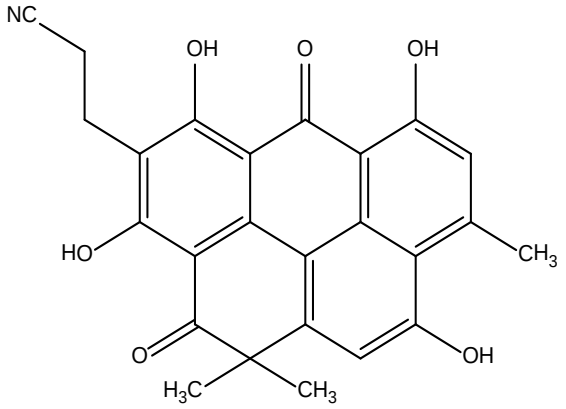

Figure 3 Structure of Cyanoxanthomycin.

3.2. Antimicrobial activities

The extract, cyanoxanthomycin was shown potent antibacterial properties against selected bacterial (Gram +ve/-ve) strains (Table 1). At the concentrations of 50 µM, it was observed between 11.6 mm zone to 19.2 mm zone of inhibition for a given bacterial cultures, compared to the standard antibiotic which shows between 7.5 mm zone to 11.2 mm zone of inhibition. For *B. subtilis*, it was inhibited by showing 11.6±0.1 mm zone of inhibition around the well which is the lowest inhibition whereas at same concentration of pure extract (cyanoxanthomycin) shows maximum inhibition 19.2 for *P. aeruginoasa.* The detailed inhibitory activity of cyanoxanthomycin on different gram-positive and negative bacteria was presented in Table 1**.**

The reported pigment has a conjugated ring system with strong intramolecular hydrogen bonds between the hydroxyl and carbonyl groups.

The polycyclic aromatic ring system belongs to the benzo [cd] pyrene structural class and is responsible for the strong fluorescence activity. The cyanoxanthomycin is likely to that of resistomycin, resistoflavin and itamycin pigments which exhibit similar structure (Kozhevina 1982). However, Resistomycin is a fluorescent pentacyclic, highly conjugated pigment and having potent antibacterial, antiviral and anti-cancer properties (Eckardt et al. 1972; Kozhevina 1982). There was no evidence of pentacyclic, cyano group fluorescent pigments from thermophiles and hyperthermophiles. Like Resistomycin, cyanoxanthomycin of *Geobacillus*, it produces yellow-orange fluorescent intensity and has selective antibacterial properties.

Table 1. Antibacterial activity of Cyanoxanthomycin.

	Organisms				
Concentration (50 µM)	*B. subtilis* (MTCC 3053) in mm	*E. coli* (MTCC 1698)	*P. aeruginoasa* (MTCC 6458)	*S. aureus* (MTCC 6908)	*Streptococcus* sp. (MTCC 9724)
Cyanoxanthomycin	11.6±0.1	16.9±0.1	19.2±0.1	18.4±0.1	17.9±0.1
Ciprofloxacin	08.2±0.1	09.1±0.1	09.6±0.1	11.2±0.1	7.5±0.1

*The values of each constituent consisted of Mean ± SE of three replicates. The concentration of 50 µM (1 µM = 429 µg/ml) was prepared by dissolving the appropriate weight of cyanoxanthomycin in sterile double distilled water (sterilized).

4. Conclusion

This is the first report of a fluorescent, pentacyclic pyrene having cyano group, a natural compound produced by a member thermophiles. We have employed a simple and easy procedure for the extraction of the fluorescent pigment from *Geobacillus* sp. Iso5. It also has characteristic antibacterial property on different bacteria. Due to lack of description on pentacyclic-derived pigments from hyperthermophiles, we believe that it has wider industrial and therapeutic applications.

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Streptomyces flora in chronically fuel oil-polluted soils and analysis of their alkane hydroxylase (*alkB*) gene by PCR

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Running Title: alkB gene in hydrocarbon-degrading Streptomyces

Abstract

This study reports the examination of the chronically fuel oil-polluted soil *Streptomyces* flora and their analysis for harboring the alkane hydroxylase gene (*alkB*) by PCR. Twenty *Streptomyces* isolates were recovered and the PCR analysis for the occurrence of *alkB* gene in the isolates 1d, 1e, 1f, 6a, 9a, and 9k showed three groups with different band size products; group 1 (G1) (215-227 bp), group 2 (G2) (334 bp), and group 3 (G3) (460-550 pb). Variation of the PCR size product of G1 from previous reports probably represents another form of *alkB* gene in these isolates.

Keywords: *alkB* gene; Biodegradation; Hydrocarbon; PCR; *Streptomyces*

1. Introduction

Attention to isolate and identify microbes prevailing in oil-polluted soils has long been reported by several investigators (Beilen *et al*., 2003; Jonathan 2003; Radwan 1998; Saadoun 2002; Saadoun 2004) and lists of hydrocarbon-degrading bacteria, actinomycetes, yeasts, fungi, and algae are therefore available. Some studies showed that *Streptomyces* flora could play a very important role in degradation of hydrocarbons (Barabas 2001; Radwan 1998; Saadoun *et al*., 2008; Saadoun and Alawawdeh 2019). For example, Barabas *et al*. (2001) have isolated 3 *Streptomyces* strains (*S. griseoflavus*, *S. parvus*, and *S plicatus*) from Burgan oil field in Kuwait with the capability to utilize *n*-hexadecane, *n*-octadecane, kerosene, and crude oil as sole carbon and energy sources. Later, Saadoun *et al*. (2008) have recovered 3 *Streptomyces* strains from soils in Jordan that are heavily polluted with crude petroleum as a result of oil spills' transportation accidents with the ability to grow on diesel as sole carbon and energy sources.

Alkane monooxygenase (*alkB*) gene is one of the key genes involved in the catabolism of alkanes and has been reported in several bacteria linked to hydrocarbon degradation including *Alcanivorax borkumensis*, *Pseudomonas aeruginosa*, *Mycobacteria* and *Rhodococcus* genera (Hamamura *et al*., 2001; Jonathan *et al*., 2003; Rojo 2013), as well as *Streptomyces* spp. (Saadoun *et al*., 2008). However, Saadoun and Alawawdeh (2019) reported the isolation of *Streptomyces* isolates from hydrocarbon-contaminated soils lacking the *alkB* gene but with a capability to degrade diesel, which implies that genes other than *alkB* gene might code for alkanes-degrading enzymes.

Saadoun (2002; 2004) has conducted studies in Jordan on isolation of different bacteria other than actinomycetes from soils polluted with oil and showed their ability to degrade diesel and short chain alkanes. Saadoun *et al.* (2008) and more recently Saadoun and Alawawdeh (2019) have shown that streptomycetes prevail in chronically oil-polluted soils of Jordan Refinery that has been historically exposed to crude or fuel oil spills for tens of years. However, these studies did not include the occurrence and recovery of streptomycetes in chronically fuel oil (refined)-polluted soils of gas stations. Therefore, the work presented here aimed to enumerate and isolate streptomycetes from soils polluted with refined fuel oil spills and to test the presence of the alkane hydroxylase gene (*alkB*) in the recovered isolates by PCR.

2. Materials and Methods

2.1. Location, sampling and sample processing.

Six gas stations in the City of Irbid/Northern Jordan with chronically fuel oil-polluted soils were selected in this study to describe the occurrence and recovery of streptomycetes in soils historically exposed to fuel spills for more than 10 years.

One fuel oil-polluted soil sample of approximately 300-400 g was collected from each station. Soil samples were taken from the 10 cm top soil, after removing the top 3 cm of the soil surface. A control soil sample was collected from a site far away from all gas stations included in this study. Samples were placed in polyethylene bags, closed

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tightly and immediately transported to the laboratory to be stored in a refrigerator at 4 ±1 °C until processed.

2.2. Processing and characterization of the sample.

Prior to analysis, collected soil samples were crushed thoroughly to remove the large debris, and then mixed and sieved through a 2 mm pore size mesh (Retsch, Haan, Germany). The soil color, pH, moisture, and total petroleum hydrocarbons were determined for all sieved samples as prescribed below.

2.3. Bacterial count, isolation and characterization of Streptomyces isolates.

Standard serial dilutional and plating techniques were used to calculate the total bacterial count (CFU/gm soil) and total *Streptomyces* count (CFU/gm soil) in all sieved soil samples.

Enumeration and isolation of *Streptomyces* spp. that were able to grow on starch casein nitrate agar (SCNA) plates were performed as described by Saadoun *et al*. (2008). Morphological and physiological characterization of all recovered *Streptomyces* isolates was performed as described by Saadoun *et al*. (2008).

2.4. Soil pH, moisture and total petroleum hydrocarbon (TPH) measurements.

To measure the soil pH, soil suspension was prepared in distilled water (1:2 w/v), and the pH was determined at 25° C (Hanna, Italy). Soil moisture was determined by drying 1 g of each soil sample at 65 °C in the oven (WTB Binder, Germany), and the difference in the weight of the sample before and after drying was considered as the moisture content. For total petroleum hydrocarbon (TPH) determination, 10 g of each soil sample were constitutively extracted with 100 ml each of hexane, dichloromethane and chloroform. The three extracted portions were all pooled and then evaporated to dryness in a fume hood (CMS, Spain) at room temperature. After solvent evaporation, the amount of residual TPH was determined gravimetrically (Saadoun *et al*., 2008; Williams *et al*., 1972).

2.5. PCR amplification of alkB gene.

2.5.1. Growth conditions.

The recovered *Streptomyces* isolates were cultured in Oxoid tryptic soy broth (TSB) (Oxoid, UK) as per Hopowood et al. (1985) and incubated at 28 °C with shaking at 140 rpm for 48 h. To check for purity of the cultures, 0.1 ml of broth was plated on starch casein nitrate agar (SCNA) plates and then incubated at 28 °C for 72 h (Küster and Williams 1964).

2.5.2. Extraction of genomic DNA from pure Streptomyces isolates.

Wizard Genomic DNA Purification Kit (Promega, USA) was used for genomic DNA extraction from approximately 40 mg (wet weight) of *Streptomyces* mycelia. DNase, RNase-Free barrier tips (Promega, USA) were used for all DNA manipulation, handling and PCR work.

2.5.3. Quantitation of the extracted DNA and estimation of its purity.

The isolated DNA was quantitated spectrophotometrically and checked for purity as per Sambrook *et al*. (1989). The extracted DNA samples were diluted in Tris EDTA (TE) buffer and measured at 260 and 280 nm wavelengths (Genesys 2, Milton Roy, USA). DNA concentration in the original sample was calculated by taking the readings at 260 nm, where 1 OD corresponds to 50 µg/ml of double-stranded DNA. However, purity of the DNA was estimated by calculating the ratio between the readings at 260 nm and 280 nm (OD_{260} /OD_{280}).

2.5.4. PCR amplification.

The following two primers; ALK-F: 5´-TCGAGCACAACCGCGGCCACCA-3´ and ALK-R: 5´ CCGTAGTGCTCGACGTAGTT-3´; (Operon Technologies, USA) described before by Saadoun *et al.* (2008) and Saadoun and Alawawdeh (2019) were used to detect the presence of *alkB* gene in all isolates. Amplification reactions were performed as described before by Saadoun *et al*. (2008) and Saadoun and Alawawdeh (2019).

2.5.5. Electrophoresis and photography.

PCR products were separated by electrophoresis on 2% agarose (Promega, USA) gels in 1X TBE buffer at 100 Volts for 1.5 h. A 100 bp DNA ladder (Promega, USA) was used to estimate the size of the PCR product. DNA bands were detected on the agarose gel by staining with ethidium bromide (EB) (Acros Organic, USA) at 0.5 µl/ml concentration. Gels were visualized and documented using Gel Doc (Bio-Rad, USA).

3. Results and Discussion

3.1. Characterization of soil samples and their Streptomyces flora.

Color, pH and moisture of the collected soil samples were determined. Results showed that the colors of the samples were black, brown or dark brown, while pH ranged from 6.89 to 7.66, and the moisture content ranged from 2.4 to 6.5% (Table 1). Total petroleum hydrocarbons (TPH) were found between 44 and 116 mg/gm of soil (Table 1). Hydrocarbons spilled on soil increase the levels of organic carbon, and may either serve as substrates for microbial growth or be toxic to microbial growth and activity (Bossert and Bartha 1984). Variation between soil-polluted samples in color, pH or the moisture content could be due to differences in levels and types of organic matter as well as differences in precipitation rates and exposure to sun. All measured values characterizing the polluted-soil samples might reflect the best conditions for microbial activities.

A control unpolluted soil sample was also evaluated for color, TPH and moisture. The sample showed a light brown color, contained 0.00 mg/g TPH and a moisture content of 4.0%. The low TPH content in the control sample is explained by lower or no exposure to fuel oil spells. This also explains the higher total bacterial and *Streptomyces* count and diversity in this sample. This observation indeed points to the inhibition effect of hydrocarbons on the growth of *Streptomyces.*

Table 1. Hydrocarbon-contaminated soil samples collected from different gas stations and their characters and *Streptomyces* content.

Sample	Gas Station	Soil sample characters					Bacterial count			*Streptomyces* color diversity	
No.	Name	Color	Weight (gm)	Moisture %	pH	TPH mg/gm	Total count CFU X10^6/ gm soil	*Streptomyces* count CFU X10^4 /gm soil	% of *Streptomyces*/ gm soil	White	Grey
1	Modern	Brown	580	4.2	7.10	56	0.87	6	6.90	3	2
2	New Irbid	Brown	335	5.5	7.32	92	0.20	2	10.0	1	1
3	Aldejany	Dark brown	682	6.5	7.66	45	0.49	6	12.24	3	3
4	Industrial City	Brown	114	4.3	7.42	50	0.20	2	10.00	1	1
5	Kuforyoba 1	Black	116	2.4	7.35	116	0.244	2	8.20	1	1
6	Boshra	Brown	220	4.1	6.89	44	0.305	3	9.83	2	1
Control	Irbid City	Dark brown	670	4.0	6.81	0.00	7.00	16	2.28	9	7

The average *Streptomyces* count ranged between 2 x 10^4 and 6 x 10^4 colony forming unit (CFU)/ gm dry soil (Table 1). However, *Streptomyces* count in the un-polluted soil was 16 ×10^4 CFU/g (Table 1). In this study, the percentage of *Streptomyces* in the polluted soils was higher than that in the control soil. Such observation might reflect how the indigenous streptomycetes get acclimated to the hydrocarbons spilled in the gas stations and might be due to the refined types of oil (benzene, diesel and kerosene), thus containing less toxic substances as compared to the crude oil that usually contains more toxic substances. On the other hand, the lower counts of *Streptomyces* in soils from New Irbid, Industrial city, Kuforyoba 1, and Boshra gas stations suggest how difficult it is for these indigenous microorganisms to prevail as a result of accumulation of TPH, which could reach toxic levels. The non-polluted control soil sample showed slightly more counts with an average of 16 ×10^4 CFU/g dry soil (Table 1). The variation in the microbial count might reflect the location of sampling as well as the amount of spilled refined-oil or concentration of pollutant. Moreover, the presence of *Streptomyces* in various habitats is affected by several factors such as pH, temperatures, nutrients and moisture (Saadoun *et al*., 2008).

The *Streptomyces* colonies observed on starch casein nitrate agar plates (SCNA) after 10 days of incubation at 27 ˚C were small (1-10 mm diameter), discrete and leathery, initially exhibited smooth surface but later developed a weft of aerial mycelium that appeared granular, powdery and velvety (data not shown). Twenty different *Streptomyces*-like isolates were recovered from the polluted soils (Table 1). However, more diverse isolates (16) were recovered from the control soil sample. The recovered *Streptomyces* isolates were phenotypically characterized into two color series only (Table 1). The occurrence of the white and the grey series was obviously more than other color series, which is in agreement with other studies by Saadoun and Al-Momani (1997) and Saadoun *et al*. (1999) who also found that these two color series occur more frequently in Jordanian soils.

3.2. Detection of alkB gene sequence in Streptomyces isolates by PCR.

The presence of *alkB* gene in the isolated *Streptomyces* was tested by designing a pair of primers by multiple alignments of several sequences from all alkane hydroxylase genes deposited in the Gene Bank (Saadoun *et al*., 2008). There are currently over 250 *alkB* gene homologues found in diverse bacterial species in which a large portion of these genes was detected in oil-contaminated environments (Wang *et al*., 2010).

PCR analysis revealed 3 groups of PCR products; group 1 (G1) isolates gave a product size of 215-227 bp, group 2 (G2) isolates gave product sizes corresponding to 334 bp, while group 3 (G3) consisting of isolates gave product sizes corresponding to 460-550 bp (Figure 1). G1 isolate 6a gave a band of 227 bp, isolate 1d gave a band of 220, while isolate 9a gave a band of 215 bp. (Fig. 1). G2 isolates (isolate 9k) gave a product with a band size of 334 bp (Fig. 1). However, G3 isolates gave a product with a higher size (460-550 bp). The isolates 1e and 1f gave a band of 544 bp each (Fig. 1). The product size of those in G2 clearly matches product size observed by Kohno *et al*. (2009) and Saadoun *et al*. (2008). Either the PCR size product of G1 did not match those observed by Kohno *et al*. (2009) or Saadoun *et al*. (2008), thus indicating another form of *alkB* gene in these isolates. This variation in product size encourages us to conduct additional molecular tests, especially for the 6a, 1d, and 9a isolates that showed a fragment size of 227, 220, and 215 bp, respectively.

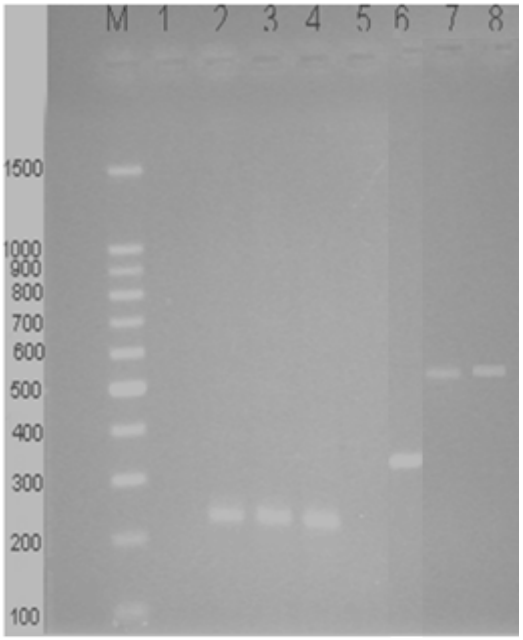

Figure 1: 2% Agarose gel electrophoresis of PCR amplification of alkane hydroxylase gene (*alkB*) from *Streptomyces* isolates with Alk primer, lane M: a 100 bp ladder, lanes 1 and 5: negative control (no DNA template), lane 2: 6a (227 bp), lane 3: 1d (220 bp), lane 4: 9a (215 bp), lane 6: 9k (334 bp), lane 7: 1e (544 bp), lane 8: 1f (544 bp).

4. Conclusion

This study highlights the power of molecular biology techniques as PCR analysis for studying the existence of alkanes-degrading *Streptomyces* flora that prevails in hydrocarbon-polluted environments. Variation of the PCR size product reported here in this study probably represents another form of *alkB* gene in *Streptomyces* isolates.

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