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EDITORIAL PREFACE

It is my pleasure to present the ninth volume of the *Jordan Journal of Biological Sciences* (JJBS) to the audience. JJBS is a refereed, peer reviewed quarterly international journal issued by the Jordanian Ministry of Higher Education and Scientific Research Support Fund in cooperation with The Hashemite University, Zarqa, Jordan. This journal publishes papers in Biological Sciences encompassing all the branches at molecular, cellular and organismal levels.

A group of distinguished scholars have agreed to serve on the Editorial Board. Without the service and dedication of these eminent scholars, JJBS would have never existed. Now, the Editorial Board is encouraged by the continuous growth of the journal and its formation into a true multidisciplinary publication. I am also honored to have the privilege of working with all members of the international advisory board served by a team of highly reputable researchers from different countries across the globe. I am also delighted with our team of national and international reviewers who are actively involved in research in different biological sciences and who provide authors with high quality reviews and helpful comments to improve their manuscripts.

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At the end of this preface, I would like to thank our readers and authors for their continuing interest in JJBS, and each member of our editorial and review boards for their continued hard work, support and dedication, which made it possible to bring another new issue of JJBS to the multidisciplinary international audience. My thanks are also extended to the Hashemite University and Jordanian Scientific Research Support Fund for their continuous support to Jordan Journal of Biological Sciences. I very much appreciate your support as we strive to make JJBS one of the most leading and authoritative journals in the field of Biological Sciences.

June,2017

Prof. Ali Z. Elkarmi
Editor-in-Chief
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Assessment of Biodegradation and Toxicity of Drill-Muds Used in an Onshore Active Field Located in Edo State, Nigeria

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Abstract

The biodegradation and toxicity of two drill-muds used in an onshore oil field located in Edo State were examined. Biodegradation of drill-muds by two bacterial and fungal isolates; *Citrobacter* sp., *Staphylococcus* sp., *Aspergillus* sp. and *Penicillium* sp. were carried in a shake flask experiment using mineral salts medium at 120 rpm for 28 days. The total viable counts were monitored and ultimate biodegradability was derived from the ratio of Chemical Oxygen Demand and Biological Oxygen Demand (BOD_5), after every four days. The water based mud was more degradable than non-aqueous based mud. This was shown by the highest total viable counts recorded in consortium amended with water based mud (126 ×103 cfu/ml), and also recording the lowest chemical oxygen demand and biological oxygen demand (45 mg/l and 0.38 mg/l, respectively). There were no significant differences ($P > 0.05$) in the degradation of the muds by the isolates. The 96 hrs and 24 hrs acute toxicity bioassay were carried out using juvenile fishes *(Tilapia guineensis)* and microorganisms (*Staphylococcus* sp.and *Aspergillus* sp.), respectively. The different concentrations were prepared for fishes (in aquaria) and microorganisms (conical flask). Mortality was recorded after 8, 24, 48, 72 and 96 hrs and 0, 2, 4, 8, 12 and 24 hr at 30 0C to assess toxicity. The 96 hr lethal concentration, 50 % (LC_{50}) of water based mud and Non-Aqueous Based Mud (NABM) were greater than10, 125 mg/l and 6000 mg/l for *Tilapia guineensis*, respectively. The 24 hr lethal concentration, 50 % (LC_{50}) of water based mud was 370 mg/l for *Staphylococcus* sp. and *Aspergillus* sp. Therefore, these selected isolates have the potential applications in the bioremediation of sites polluted by these drill-muds. Also, in the interest of the environment, oil exploration and production companies operating in Edo State and other parts of Nigeria should strictly adhere to the use of non-toxic and biodegradable drilling muds during exploration activities.

Keywords: Toxicity, biodegradation, drilling muds, bacteria, fungi.

1. Introduction

In oil and gas operations, drilling fluids, also referred to as drilling muds, are used to lubricate and cool the drilling apparatus, transport drill cuttings to the surface and seal off porous geologic formations (Odokuma and Ikpe 2003; Okoro, 2011; Imarhiagbe and Atuanya, 2013; Linjun, 2013). Drilling fluids typically consist of bentonite and a range of additives mixed with fresh water or hydrocarbons. The two primary types of drilling muds are water based muds and non-aqueous based muds (Mairs et al., 1999). Water based muds consist of water mixed with bentonite clay and additives, such as barium sulfate (barite); they are used for most types of drilling. The non-aqueous drilling muds (NABM) comprise all non-water and non-dispersible based muds and they include Oil Based Muds (OBM), Low Toxicity Mineral Based Muds (LTMBM), Enhanced Mineral Oil Based Muds (EMOBM) and Synthetic Based Muds (SBM) (Mairs et al., 1999; Anwuli, 2011; Ogeleka and Tudararo-Aherobo, 2013) and are mostly used in offshore wells or other water sensitive formations.

According to Odokuma and Akpanah (2008), in Nigeria, drilling muds and cuttings are sometimes discharged into fills and from where they over flow into nearby farms and rivers. Small amounts are re-injected into special Cutting Re-Injection (CRI) wells while lesser amounts are treated in Thermal Desorption Units (TDU). The three basic types of drilling muds are water based mud, oil based mud and synthetic based mud (Okoro, 2011). Researchers have abundantly shown that drilling muds additives may contain toxic substances such as heavy metals, hydrocarbons, biocides, chromate, organic polymers and trace elements that have the tendency to bioaccumulate and interfere with normal biological activities of organisms (Odokuma and Ikpe, 2003; Odokuma and Akponah, 2008; Vincent-Akpu et al.,2010). Studies, according to Engelhard et al. (1989) and Vincent-

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Akpu et al. (2010) have been conducted with various drilling fluids in the North Sea using mortality as the criterion for determining their effects on the biota. Odokuma and Ikpe (2003) observed that water based muds were more biodegradable than oil based muds. They ascribed this observation to the greater toxicity of oil based muds.

According to Ogeleka and Tudararo-Aherobo (2013), an average of 7000 to 13000 bbl of waste per well, composing approximately 1400 to 2800 bbl of drill cuttings depending on the depth and diameter of the well when water based muds were use during drilling, with an average volume estimation of 2000 to 8000 bbl waste per well when oil based mud was used. In most oil producing countries, drill-muds and drill-cuttings are discharged on site (Ogeleka, and Tudararo-Aherobo, 2013) including Nigeria (Anwuli, 2011; Odokuma and Akponah, 2008); the improper disposal of drilling muds and cuttings have been found to pose a significant stress on the ecosystem of the receiving environment (Odokuma and Ikpe, 2003). Edo State is one of the states in the Niger-Delta region of Nigeria, playing host to several oil and gas exploration companies. The present work, therefore, assess the biodegradation and toxicity of the two main classes of drilling muds (water based mud and non-aqueous based mud) now commonly employed in drilling operations in this part of Niger-Delta region in Nigeria..

2. Material and Methods

2.1. Source of Test Isolates

The test isolates, employed in the preset study, were earlier isolated from drill cuttings obtained from a land rig situated in an oil-producing community in Edo State (Imarhiagbe, 2012).

2.2. Collection of Drilling-Muds

The drilling-muds used in the present study were collected from the drilling site located in Edo State oil-producing community (the Geographic Position System (GPS) coordinate of the well was E: 350020.000 m, N: 229477.600 m) and was coded as non-aqueous based mud and water based mud. Samples were transported to the laboratory aseptically for evaluation, in labeled plastic containers.

2.3. Monitoring the Biodegradation

Biodegradation of drill muds by microorganisms were carried in a shake flask experiment using mineral salts medium. The mineral salt medium composed of the following: MgSO4.7H2O, 0.42 g/l, KCl, 0.30 g/l, KH2PO4, 0.8 g/l, K2HPO2, 1.3 g/l, NaNO3, 0.42 g/l and agar 15 g/l (Okpokwasili and Okorie, 1988). Two predominant drill cuttings isolated bacteria (*Citrobacter* sp., *Staphylococcus* sp.) and fungi (*Aspergillus* sp. and *Penicillium* sp.) were selected for this test. One hundred and fifty milliliters (150 ml) of the mineral salt medium was dispensed into five (5) different 250 ml conical flasks in duplicate and 10 ml of each drilling mud was added. Bacterial and fungal inoculants for this experiment was prepared by suspending a loopful of each isolate in 2 ml of mineral salt medium. Each organism was introduced into separate conical flask, while a consortium of the bacterial and fungal isolates was transferred into separate conical flasks. The control conical flask remained uninoculated. All flasks were incubated at room temperature on a rotary shaker operating at 120 rpm for 28 days. The total viable counts were monitored and ultimate biodegradability was derived from the ratio of Chemical Oxygen Demand and Biological Oxygen Demand (BOD_5), after every four days.

2.4. Toxicity Assay

The 96 hr acute toxicity bioassay was carried out using juvenile fishes (*Tilapia guineensis)* according to Organization for Economic Co-operation and Development [OECD] (1995). Non-aqueous base mud and water base mud were separately prepared into six different aquaria while the seventh was used as control, without the test chemical. The different concentrations, 1000 mg/l, 4000 mg/l, 5000mg/l, 6000 mg/l, 8000 mg/l and 10000 mg/l of the test chemicals were prepared. The fishes were distributed randomly in batches of ten per concentration into the seven aquaria. The organisms were not touched with bare hands during selections so as to avoid stress due to handling. The fishes were exposed to an initial period of acclimatization. The experiment was observed hourly for any death. Mortality was recorded after 8, 24, 48, 72 and 96 hours. Method used for 24 hrs lethal toxicity assay was adapted from Odokuma and Ikpe (2003) using bacteria (*Staphylococcus* sp.) and fungi (*Aspergillus* sp.). A loopful of the bacterial and fungal cells were collected from their individual slants and dislodged in 10 ml of normal saline, and then allowed to stand for few hours. An approximate cell dilution was chosen (*Staphylococcus* sp was 4.0x103 cfu/ml and *Aspergillus* sp was 3.3 x103 cfu/ml). Thereafter, 10 mg/l, 50 mg/l, 100 mg/l, 150 mg/l, 200 mg/l and 250 mg/l concentration of the drilling muds were prepared, respectively, in 250 ml conical flasks. The control was distilled water. The mixture was vigorously shaken for even mixing. One milliliter of each set-up including control was plated out at 0, 2,4, 8,12 and 24 hr at 30 ^{0}C to determine the viable cells and to assess toxicity. While the bacterial isolate was plated on nutrient agar, the fungal isolate was plated on potato dextrose agar incorporated with chloramphenicol. At the end of incubation, viable cells were counted and recorded. The lethal concentration (LC 50/24) values were extrapolated from the graph of mortality against concentration.

3. Results

3.1. Monitoring the Biodegradation

Tables 1(A-B) and figures 1(A-D) show the ability of single cultures of *Citrobacter* sp, *Staphylococcus* sp., *Aspergillus* sp., *Penicillium* sp., and their mixed cultures to degrade water based mud and non-aqueous based mud as depicted in their Chemical Oxygen Demand (COD) and Biological Oxygen Demand (BOD_5) graph trends. The biodegradation potentials of the above isolates were monitored over a period of twenty-eight (28) days by assessing their total viable counts, chemical oxygen demand and biological oxygen demand. From the results, cultures containing consortiums (mixed isolates) showed remarkable biodegradation potential when compared to single isolates; and the water based mud showed a higher degree of biodegradation when compared with non-

aqueous based mud. The results showed that the highest total viable counts were observed in culture medium amended with water based mud (126x 10^3 cfu/ml and 10.5x 10^3 cfu/ml) for bacterial and fungal counts, respectively. Whereas, single cultures had their maximum growth peaks at day 16, mixed cultures had their peaks at day 24, 28(bacterial counts) and day 20 (fungal counts).

Table 1a. Total Viable Counts of Bacterial Isolates in Culture Medium Amended With Drilling Mud (10^3 Cfu/Ml)

	initial		Day 4		Day 8		Day 12		Day 16		Day 20		Day 24		Day 28	
	WBM	NABM	WBM	NABM	WBM	NABM	WBM	NABM	WBM	NABM	WBM	NABM	WBM	NABM	WBM	NABM
Citrobacter sp.	3.5	3.5	4.7	4.4	5.3	4.4	45.1	10.0	48.0	15.2	47.2	32.0	60.0	15.3	19.0	11.0
Staphylococcus sp.	4.1	3.7	5.1	4.9	5.3	5.3	47.0	25.0	56.0	52.0	45.0	38.0	41.0	43.0	36.0	25.0
Citrobacter + *Staphylococcus* sp.	4.5	4.0	4.5	4.6	5.9	5.7	82.0	62.0	90.0	64.0	99.0	67.0	112.0	83.0	126.0	75.0
Control	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Over all mean values. Control contained no isolate.

Table 1b. Total Viable Counts Of Fungal Isolates In Culture Medium Amended With Drilling Mud (10^3 Cfu/Ml).

	initial		Day 4		Day 8		Day 12		Day 16		Day 20		Day 24		Day 28	
	WBM	NABM	WBM	NABM	WBM	NABM	WBM	NABM	WBM	NABM	WBM	NABM	WBM	NABM	WBM	NABM
Aspergillus sp.	3.0	3.0	3.4	3.1	4.1	3.4	5.2	3.8	5.8	4.4	6.5	5.1	6.6	5.1	6.6	4.9
Penicillium sp.	2.9	3.0	4.6	4.9	4.8	5.1	5.2	5.5	7.0	5.7	7.0	6.0	7.1	6.0	7.2	5.7
Aspergillus sp. + *Penicillium* sp.	3.0	3.0	6.3	6.3	7.5	7.5	8.9	8.9	9.1	9.1	9.1	9.1	9.1	9.1	7.0	7.0
Control	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Over all mean values. Control contained no isolate.

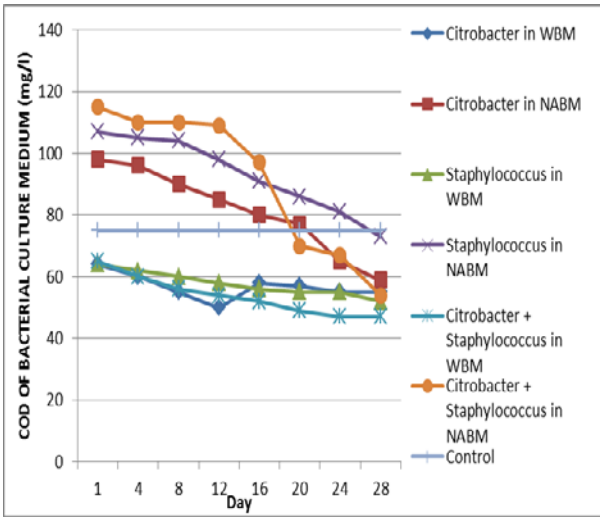

Figure 1A. Chemical oxygen demand of bacterial culture medium amended with drilling mud

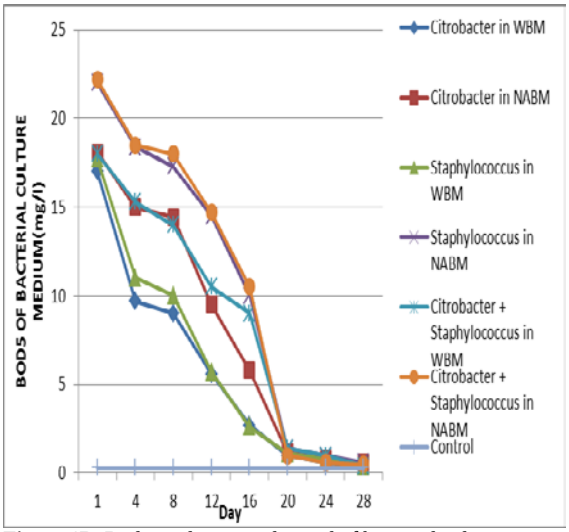

Figure 1B. Biological oxygen demand of bacterial culture medium amended with drilling mud

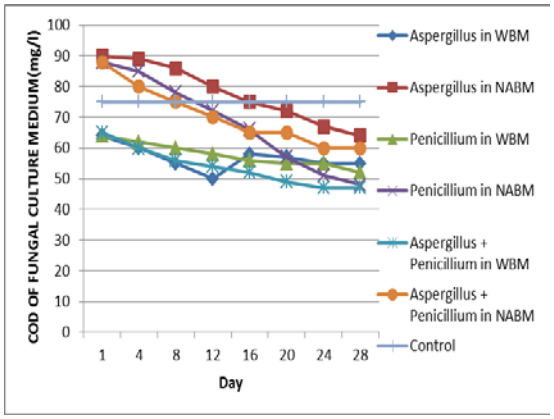

Figure 1C. Chemical oxygen demand of fungal culture medium amended with drilling mud

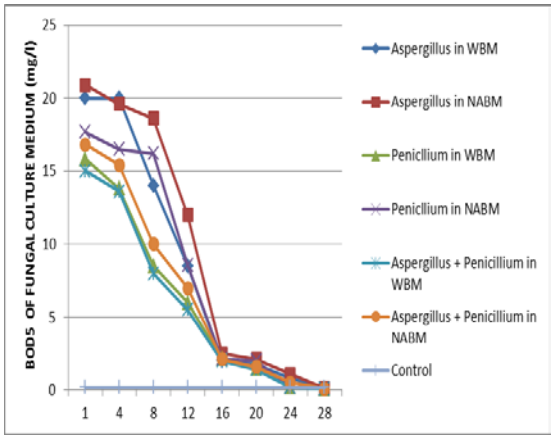

Figure 1D. Biological oxygen demand of fungal culture medium amended with drilling mud

3.2. Toxicity assay

The results of the toxic effect of the drilling muds on selected test organisms are presented in tables 2-3 (A-C).

Table 2 shows lethal concentration (LC_{50}) of the drill muds used at the location. The results showed that the non-aqueous based mud was more toxic to the test organisms than the water based mud. The 96 hours LC_{50} of WBM was greater than 10,125 mg/l while the 96 hrs LC_{50} of NABM was greater than 6000 mg/l for *Tilapia guineensis*. The 24 hrs LC_{50} of WBM was greater than 370 mg/l for *Staphylococcus* sp. and 300 mg/l for *Aspergillus* sp., and 24 hrs LC_{50} of NABM was 280mg/l and 255 mg/l for *Staphylococcus* sp. and Aspergillus sp. respectively. The effective dead time (tables 3 A-C) of the test organisms at different concentrations of the drilling muds revealed that at concentrations 250 mg/l, death of *Staphylococcus* sp. and Aspergillus sp. occurred within 12 to 24 hours of exposure to water-based mud with mortality rate of 1.38x103 cfu/ml and $1.5x10^2$ cfu/ml, $5.2x10^2$ cfu/ml, respectively; while at concentrations 100 mg/l of non-aqueous based-mud, death occurred in less than 2 hrs of exposure with mortality rate of 4.7 x 10^2 cfu/ml and $7.5x10^2$ cfu/ml, respectively. The effective time of *Tilapia guineensis* at varied concentrations of water based mud and non-aqueous based mud was observed to be 48hrs and 96 hrs of exposure, respectively.

Table 2: Lethal Concentration (Lc_{50}) Of Drill Muds Used in the Drilling Muds.

DRILLING MUD TYPE	*Staphylococcus* sp.(24hrs LC_{50}) mg/l	*Aspergillus* sp. (24hrs LC_{50}) mg/l	Tilapia (96hrs LC_{50}) mg/l
WBM	>370	>300	>10, 125
NABM	>280	>255	>6000

Values represent means of duplicates

Table 3 A. Effective Time for Concentration of Drill Muds Toxicity Test on *Staphylococcus* Sp.

	0hr		2		4		8		12		24		TOTAL MORTALITY		% MORTALITY	
	WBM	NABM	WBM	NABM	WBM	NABM	WBM	NABM	WBM	NABM	WBM	NABM	WBM	NABM	WBM	NABM
Control	-	-	-	-	-	-	-		-	-	-	-	-	-	0	0
10 mg/l	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0	0
50 mg/l	-	-	-	-	-	$1.5x10^2$	-	$1.0x10^2$	-	$1.0x10^3$	-	$1.7x10^2$	-	$1.42x10^3$	0	35.5
100 mg/l	-	$4.7x10^2$	-	$1.0x10^3$	-	$1.5x10^3$	-	-	-	-	-	-	-	$2.97x10^3$	0	74
150 mg/l	-	$7.0x10^2$	-	$2.0x10^3$	-	$6.9x10^2$	-	-	-	-	-	-	-	$3.39x10^3$	0	85
200 mg/l	-	$4.0x10^3$	-	-	-	-	-	-	-	-	-	-	-	$4.0x10^3$	0	100
250 mg/l	-	$4.0x10^3$	-	-	-	-	-	-	-	-	$1.38x10^3$	-	$1.38x10^3$	$4.0x10^3$	35	100

An inoculum of 4.0 x 10^3 cfu/ml was introduced into each solution; (-) means no mortality; WBM = water Based Mud; NABM = Non-aqueous Based Mud.

Table 3b. Effective Time for Concentration of Drill Muds Toxicity Test on *Aspergillus* Sp.

	0hr		2		4		8		12		24		TOTAL MORTALITY		% MORTALITY	
	WBM	NABM	WBM	NABM	WBM	NABM	WBM	NABM	WBM	NABM	WBM	NABM	WBM	NABM	WBM	NABM
Control	-	-	-	-	-	-	-		-	-	-	-	-	-	0	0
10 mg/l	-	-	-	-	-	$2.5x10^2$	-	$1.0x10^2$	-	$1.7x10^3$	-	$1.0x10^3$	-	$3.05x10^3$	0	92.4
50 mg/l	-	-	-	$1.0x10^2$	-	$1.0x10^3$	-	$1.0x10^2$	-	$1.8x10^2$	-	-	-	$2.3x10^3$	0	69
100 mg/l	-	$7.5x10^2$	-	$1.0x10^3$	-	$2.0x10^3$	-	-	-	-	-	-	-	$2.85x10^3$	0	86
150 mg/l	-	$7.0x10^2$	-	$2.5x10^3$	-	-	-	-	-	-	$1.9x10^2$	-	$1.9x10^2$	$3.3x10^3$	5.76	100
200 mg/l	-	$2.0x10^3$	-	$1.3x10^2$	-	-	-	-	$1.0x10^2$	-	$2.0x10^2$	-	$3.0x10^2$	$3.3x10^3$	9.1	100
250 mg/l	-	$3.3x10^3$	-	-	-	-	-	-	$1.5x10^2$	-	$5.2x10^2$	-	$6.7x10^2$	$3.3x10^3$	20	100

An inoculum of 3.3×10^3 cfu/ml was introduced into each solution; (-) means no mortality; WBM = water Based Mud; NABM = Non-aqueous Based Mud.

Table3 C. Effective Time for Concentration of Drill Muds Toxicity Test on *Tilapia Guineensis* (Fingerlings).

	8hr		24hr		48hr		72hr		96hr		TOTAL MORTALITY		% MORTALITY	
	WBM	NABM	WBM	NABM	WBM	NABM	WBM	NABM	WBM	NABM	WBM	NABM	WBM	NABM
Control	-	-	-	-	-	-	-		-		-		0	0
1000 mg/l	-	-	-	-	-	-	-		-		-		0	0
4000mg/l	-	-	-	-	-	-	-		-		-		0	0
5000mg/l	-	-	-	-	-	-	-		-	2	-	2	0	20
6000mg/l	-	-	-	-	-	1	-	2	-	3	-	6	0	60
8000mg/l	-	-	-	-	-	2	-	3	2	3	2	8	20	80
10000mg/l	-	-	-	-	-	3	-	4	4	3	4	10	40	100

Ten (10) fingerlings were introduced into each tank; (-) means no mortality; WBM = water Based Mud; NABM = Non-aqueous Based Mud

4. Discussion

In the present study, a twenty-eight day monitoring of the biodegradation potential of *Citrobacter* sp., *Staphylococcus* sp., *Aspergillus* sp. and *Penicillium* sp. revealed a consistent increase and decrease of the total viable counts (cfu/ml). The results showed that the highest total viable counts were observed in culture medium amended with water based mud ($126x\ 10^3$ cfu/ml and $10.5x\ 10^3$ cfu/ml) for bacterial and fungal counts, respectively. The presence of relatively toxic oil in the liquid phase of non-aqueous base mud may have contributed to its low microbial counts; this was contrary to that of water base (Odokuma and Ikpe 2003). The highest total viable counts (cfu/ml) were recorded for the broth batches containing consortium of isolates (*Citrobacter* sp. + *Staphylococcus* sp WBM broth; *Citrobacter* sp.+ *Staphylococcus* sp in NABM broth; *Aspergillus* sp + *Penicillium* sp in WBM broth and *Aspergillus* sp + *Penicillium* sp in NABM broth). The experimental results also showed that while axenic (single) cultures had their maximum growth peaks at day 16, mixed (consortium) cultures had their peaks at day 24, 28(bacterial counts) and day 20 (fungal counts). This is in line with previous works that had suggested that mixed microbial cultures are better degraders of organic pollutants (Okpokwasili and Okorie, 1988; Odokuma and Ikpe, 2003). Thus, flasks containing consortium showed marked biodegradation potential in comparison with flasks containing single isolates. Enhanced degradation observed by the microbial consortium in the present study may be attributed to the fact that an organism may have acted as primary utilizer, utilizing substrate molecules while the other acted as secondary utilizer, utilizing the breakdown products of substrate after initial attack by primary utilizer (Okpokwasili and Okorie, 1988). Statistical analysis revealed no significant differences ($P > 0.05$) in the degradation of the muds by the isolates. According to Okerentugba and Ezeronye (2003) the isolation of certain oil-degrading micro-organisms in a polluted environment is an indication that these micro-organisms are the active degraders of that environmental pollutant. Alan (2006) earlier showed that the authochthonous aerobic microbial populations have the capability to utilize the non-aqueous base fluid as their sole carbon and energy sources. It therefore showed that these selected isolates have potential applications in the bioremediation of sites polluted by water based mud and non-aqueous based mud.

The eco-toxicity analysis of these two types of drilling muds with reference to their composition showed that the non-aqueous based mud was more toxic to the test organisms than the water based mud (Tables 2-3) and may be ascribed to their chemical composition. The lethal concentration, 50 % (LC_{50}) of the drilling muds again buttressed the obvious fact that water based muds are relatively less or non-toxic when compared with non-aqueous base muds. From the study, lethal concentration, 50 % (LC_{50}) of water base mud was greater than 10, 125 while that of the non-aqueous base mud was greater than 6000. The increase in percentage mortality as concentration of drilling muds increased over time of exposure in this study, which is in agreement with previous findings (Ekpo and Ekanem, 2000; Vincent-Akpu, 2010). Vincent-Akpo (2010) and Ogeleka, and Tudararo-Aherobo (2013) had earlier shown a positive correlation between

toxicant concentrations (drilling muds) and fish mortality rate in treated tanks.

Several authors had shown that the toxicity of drilling muds may be linked to their chemical entities such as the base fluid types (Ekpo and Ekanem, 2000; Odokuma and Ikpe 2003), concentration, water solubility (Odokuma and Akponah, 2008) and genetic constitution of the organism (Dutton et al., 1990). Neff et al. (2000) had attributed the toxicity of drilling muds to their hydrocarbon content. The low hydrocarbon content along with other chemical compositions of drilling muds are responsible for their toxicity and also the fact that synthetic based mud does not disperse in water is an additional contributing factor to its toxicity and low biodegradation.

5. Recommendations

Oil exploration and production companies operating in Edo State, Nigeria and other parts of the world, should strictly adhere to the use of non-toxic and biodegradable drilling muds in the interest of safeguarding their immediate environment. Also, the legislative ban on the use of toxic oil-based mud should be properly monitored and enforced by their appropriate regulatory agencies.

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First Record of Leech *Dina Punctata* (Annelida: Erpobdellidae) from Lesser Zab River in Northern Iraq: Morphological and Molecular Investigation

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Abstract

A total of 17 specimens of the leech *Dina punctata* were collected from Lesser Zab River in Zirandul region near Qashqoli village during the period from July to October 2015. Specimens were examined either live using dissecting microscope, or after being fixed in 5% formaldehyde. Specimens used for molecular analysis of genomic DNA were fixed and preserved in absolute ethanol. Following DNA extraction, region of 28S rDNA was amplified by Polymerase Chain Reaction (PCR), and the nucleotides order was determined using genetic analyzer. Morphological and morphometric futures as well as molecular analysis showed that the collected specimens belonged to *D. punctata*. This is the first record for this species in Iraq.

Keywords: *Dina punctata*, 28S rDNA marker, DNA sequencing.

1. Introduction

Leeches belonging to the family Erpobdellidae constitute an important part of the freshwater benthic fauna of the Northern Hemisphere. Most erpobdellid species are predators on small invertebrates (Dall, 1983; Toman and Dall, 1997, Siddall, 2002). The seven genera of the family are chiefly characterized by two taxonomic characters: the presence or absence of a pre-atrial loop formed by paired male gono-ducts and the type of annulation (Sawyer, 1972). External characters like body shape, size, color and color patterns can be very variable depending on the method of fixation (Nesemann and Neubert, 1994). However, the phylogenetic relationship among genera of Family Erpobdellidae has been assessed based on morphological characters as well as molecular analysis of mitochondrial cytochrome c oxidase subunit I, mitochondrial 12S rDNA and nuclear 18S rDNA (Siddall, 2002). In the present report, the genera *Dina, Mooreobdella, Nephelopsis and Trocheta* are formally synonymised under the genus *Erpobdella*, the type genus of the family. In the present report, genus *Dina* is retained as it is still being used by many investigators (See Jeug (2008), Kutschera (2010) and Ahmad *et al*. (2015))**.**

The three most abundant and species-rich genera of erpobdellid leeches are *Erpobdella, Dina* and *Trocheta.* These have traditionally been distinguished by their annulation pattern. *Erpobdella* has five unsubdivided annuli per somite; in *Dina* the last annulus is widened and subdivided once, and in *Trocheta*, the last and often also the first annulus is further subdivided. Furthermore, the annulation pattern has proved to be inappropriate for the diagnosis of *Dina* (Neubert and Nesemann, 1995; Trontelj and Sket, 2000; Sket and Trontelj, 2008).

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The present study reports for the first time on the occurrence of the leech *D. punctata* in Lesser Zab River, near Erbil City, northern Iraq. The leech was identified on the basis of morphological criteria and molecular analysis of 28S rDNA.

2. Materials and Methods

2.1. Study Site and Parasite Materials

In the present study, 17 samples of leeches were collected from Lesser Zab River from one site Zirandul region about 2 km before Qashqoli village, 109 km from Erbil City during the period July to October 2015. In the laboratory, 9 specimens were either examined alive using a dissecting microscope, or 5 specimens fixed in 5% formaldehyde for dissection further examination. Three specimens were fixed and preserved in absolute ethanol for molecular analysis.

2.2. DNA Extraction, PCR Amplification and Nucleotide Sequencing

Genomic DNA from leech specimens was prepared using a DNA extraction kit (GeNet Bio, KOREA) and following the manufacturer's instructions with minor modification. Briefly, leech specimens were macerated in mortar and pestle, and the contents were transferred into sterile tubes containing 200-250 µL tissue lysis buffer and kept in incubator for 4 hours. Qualification and quantification of DNA concentration was performed by using Nano Drop (ND- 1000, USA). Samples of genomic DNA with (A260–A320) / (A280–A320) ratio more than 1.7 and outputs more than 30 ng/µL were obtained.

A region of 28S rDNA was amplified by polymerase chain reaction (PCR). The primers were universal, forward primer C1 (ACCCGCTGAATTTAAGCAT at position 25), and reverse primer C3 (CTCTTCAGAGTACTTTTCAAC at position 390), as designed and selected by Mollaret *et al.* (2000). PCR reaction and conditions were performed using MJ Research, Applied Biosystem (AB) thermal cycler. Fifty µL reaction mixture was prepared in PCR tubes containing 2.5 µL DNA templates, 25 µL OnePCR^{TM} master mix (GENEDIREX, KOREA), 1 µL for each primer and 20.5 µL double deionized water (ddH_2O). The cycling conditions comprised of initial denaturation at 94˚C for 5 min, 35 cycles of denaturation at 94˚C for 45 sec, annealing temperatures at 51˚C for 45 sec and extension at 72˚C for 45 sec, and final extension at 72˚C for 5 min. Agarose gel electrophoresis was employed to check the efficiency of PCR reactions. The samples were prepared and run in 2% gel of agarose then stained with SYBR green that makes the DNA visible under UV light.

The ABI 3130X nucleotide sequence analyzer (SINGAPORE) was used to find nucleotides order of 28S rDNA from the specimens. The PCR fragments of the specimens were excised from the agarose gel and used as a source of DNA template for sequence specific PCR amplification.

3. Results

3.1. Morphological Characterization

Thick and large leeches up to 75 mm length, with colors that vary from dark brown or reddish brown to greenish with one pair of dirty paramedian stripes and yellowish spots were arranged transversally on each annulus. As for annulations, the somites consist of four short, and one long, annuli, with annulation formula (b1, b2, a2, b5 and b6), b6 annuli is larger than the other and divided by a shallow furrow (c11, c12). Genital pores separated by 1.5 to 2 annuli; male gonopore in the furrow of XII b2/a2, female pore in XII b5.b6. Atrium thick with curved cornua and simple coiled ends (Nesemann, 1993).

Nine specimens were used to determine the morphometric features of the collected leeches. The leeches were greenish in color with bright transverse yellow spots in life specimens (Fig. 1: A; B; C). Their dimension ranges and (means) were as follows: length, 20.3-70.7 mm (50.6 mm), width, 6.2-8.9 mm (7.5 mm), atrium, 2.1-2.3 mm (2.25 mm) on smoite XII (reaching from XI/XII to XII b2/a2) (Fig. 1 E), pseudognaths highly developed, 1.8-2.2 mm (1.92 mm) (Fig. 1 A; C). Morphometric features conform well to *Dina punctata* descriptions as per Nesenann (1993).

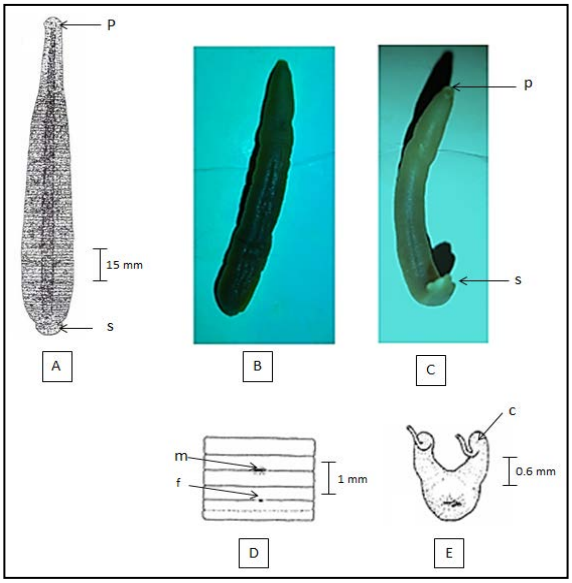

Figure 1. *Camera Lucida* drawings and photomicrographs of a leech specimen that were identified as *Dina punctata*.

A. Camera lucida drawings of the leech (Dorsal side), showing pseudognaths (P) and posterior sucker (S).
B. Photomicrography of the leech (Dorsal side).
C. Photomicrography of the leech (Ventral side).
D. Male (m) and female (f) gonopores on somite XII (Ventral view).
E. Atrium with cornua (c) (Ventral view).

3.2. Molecular Characterization

The sequence from 28S rDNA of *leech specimens* was made of 300 bp (amplified fragment was 365bp, while after sequencing 65 miss-nucleotides were excluded, related to quality of sequencing analysis) and put to BLAST then compared with other stored species of *Dina* sequences from GenBank database. The BLAST results indicated that the query sequence was more than 99% identical to *D. punctata* (Fig. 2 and 3).

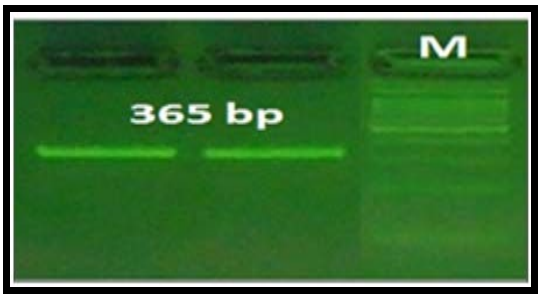

Figure 2. The result of 2% agarose gel stained with SYBR green of 28S rDNA of leech specimens following molecular analysis.

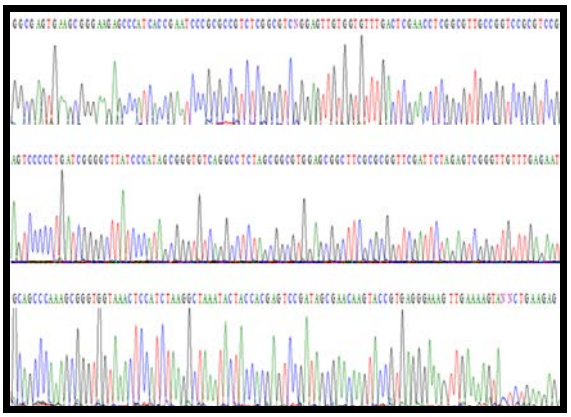

Figure 3. The chromatography sequence result of 28S rDNA sequence of leech specimens which proved to be more than 99% identical *to Dina punctata.*

4. Discussion

The morphological characters, body part measurements and colorations of the examined specimens conform to the descriptions of *D. punctata* recorded by Moore (1939), Nesemann (1993) and Ahmed *et al*. (2015). Previously, only one species of the present genus, *D. lineata* was recorded in Kurdistan Region, Iraq from Greater Zab River by Ali and Jaweir (2013). Since, there are no previous reports for this species in Iraq, the present records regarded as the first for *D. punctata* in this country.

Dina lineata was recorded from Spain and two assortments notata and punctata was recognized (Nesemann, 1993). Johansson (1927) described the new assortment *punctata*, but subsequent authors did not segregate the two assortments. Ahmed *et al*. (2015) reported that the scientific classification of the genus *Dina* in the western Mediterranean requires revision. Additionally, Minelli (1979) reported that *D. lineata* recorded from Italy is most likely *D. punctata*. Indeed, Jueg (2008) indicated that *D. punctata* in the Iberian Peninsula is exceptionally regular and *D. lineata* is absolutely truant. This investigator recommended that *D. lineata* reported from the Iberian Peninsula by García-Más and Jiménez (1984) and García-Más *et al*. (1998) was actually *Dina punctata*. Furthermore, Nesemann and Neubert (1994) described *D. punctata maroccana* as another subspecies from Morocco and they suggested that some of the specimens described as *Dina lineata* by Moore (1939) from Morocco can be considered to be co-unspecific to their new subspecies. Ahmed *et al*. (2015) thought that *D. punctata maroccana* may be synonymous with *D. punctata*. The taxonomic status of D. lineata and the geographic conveyance of *D. punctata* stay unverifiable till (Nesemann, 1993; Ahmed *et al*., 2015).

Most external characters and dorsal coloration of *Dina punctata* and *Dina stschegolewi* are similar (Nesemann, 1993). In addition to this, for proper identification of some taxa the cocoons need to be examined. Indeed, Kutschera (2010) mentioned that the new described species *Trocheta intermedia* a leech from Germany resembles the taxon *D. punctata* from Switzerland, but they differ in colorations of cocoons, and in most cases collection of these cocoons is difficult.

Thus, there is a need for more reliable methods including molecular analysis to identify species belonging to genera of Family Erpobdellidae (Siddall, 2008). *D. punctata* species is molecularly well distinguished from the other available species of *Dina.* The primary sequence analysis using universal primers of studied specimens revealed that the leech from northern Iraq belongs to species *D. punctata* (Fig. 3). Its rDNA conforms to the same rDNA sequence fragment marker, available at the GeneBank in National Center for Biotechnology Information (NCBI).

In conclusion, that is the first record for the existence of *Dina punctata* in Iraq. The morphological characters, body parts and colorations of the specimens and DNA sequence based analysis revealed the identity of *D. punctata.*

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Neural Network Based Prediction of 3D Protein Structure as a Function of Enzyme Family Type and Amino Acid Sequences

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Abstract

Prediction of dihedral angles from amino acid sequences based on the neural network to predict protein structure is promising in the field of bioinformatics. The present proposed study presents a prediction tool for 3-Dimensional (3D) protein structure as a function of enzyme family types and amino acid sequences. 11 different families of enzymes were investigated amounting to 97 enzymes in total. Correlation of sequence with geometry coordinates as a function of amino acid descriptors and family class were generated through a neural network to predict coordinates. The structural-features of dissected triplets show significant influence on family type. R-values for the inter-family dataset as well as feature selection were not satisfying. In contrast, R-values around 0.8 were achieved in the case of intra-family prediction. Furthermore, about 55 % of features were eliminated with a limited negative influence of 13% on the R-value. We believe that the present study provides a promising prediction method that advance computational methods in bioinformatics, especially to predict 3D protein structure as a function of enzyme family type and amino acid sequences. However, intra-family prediction probability is higher while using only one type of analysis based on the dihedral angles of turn structures of enzyme families.

Keywords: Neural Network, 3D Protein, prediction, Enzyme Family, Amino acid sequences.

1. Introduction

The complete genetic blueprint of a human being is now available for implementing new effective therapeutic strategies (Piccoli *et al*., 2013; Singh *et al*., 2013; Zini, 2005). Human DNA information is a powerful tool used to explore the role of genetic codes in pathogen formation and in the development of several diseases that form the majority of health problems worldwide, like cancer, diabetes, cardiovascular and others(Csermely *et al*., 2013; Mathkour and Ahmad, 2010; Oakley *et al*., 2008). This valuable trove of data has limitations in understanding higher order protein structures and particularly in translating protein molecular functionality from linear codes (Friedberg, 2006).

The knowledge of predicting three-dimensional structure of a protein can be used, on one hand, in drug design and in understanding biological mechanisms of protein function. X-ray, NMR, and, to some extent, electron microscopy are methods used to measure protein folding and surface topography. These methods however are limited in decoding the structure of many vital proteins classes (Pavlopoulou and Michalopoulos, 2011).

On the other hand, structure prediction from amino acid sequence requires the development of complex algorithms and is dependent on the millions of data points extracted experimentally to solve protein structures (Mills *et al*., 2015). In addition, algorithms should also be able to predict newly discovered or yet unrevealed structures (Kryshtafovych and Fidelis, 2009).

Deciphering algorithms of how protein structure is predicted as a function of primary sequence is no longer a purely academic problem, but can be used as a powerful method leading to effective drug design (Ahsanullah *et al*., 2012; El-Dahshan *et al*., 2014; Pavlopoulou and Michalopoulos, 2011).

Although the level of complexity between the primary sequence and final structure is relatively high, integrity and synchronization of protein building blocks, i.e., utilization of amino acid sequence to determine the folding process and the final 3D structure (Babu *et al*., 2011; Dokholyan, 2006; Liu *et al*., 2011). However, protein structure has been reported to be

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classified on three levels: primary, secondary, and tertiary structure (Zhang, 2009).

The first level consists of the sequence of amino acids making a linear structure, whereas the secondary structures depict the kinks and folding process where alpha helices and beta sheets are formed (Sikder and Zomaya, 2005). Although, the tertiary structure can be understood by the means of algorithms to be generated that includes the "turns" weave secondary structure fragments and lay the orientation of a whole ribbon in 3D space (Kryshtafovych and Fidelis, 2009).

One of the challenges in science is to predict the coordinate of these structure fragments (Grana *et al*., 2005; Liwo *et al*., 2011). In the present paper, "turns" that form the tertiary structure extracted from several enzyme families were investigated and correlated to structure of the description of the 3D structures of the studied proteins.

Correlation of sequence with geometry coordinates as a function of amino acid descriptors and family class were generated through a neural network tool to predict spatial configurations of the acids.

2. Methods

2.1. Database Mining

Eleven different families of enzymes were selected and investigated including EC 1.1.1.X (were X = 1, 2, 3, 8, 9, 10, 14, 17, 18, 21, or 22) with a grand total of 97 enzymes.

Structures were extracted from the Expert Protein Analysis System (ExPASy) a bioinformatics resource as well as from the protein data bank (Artimo *et al*., 2012). Secondary structures consisting of α-helices and β-strands were removed from the Protein Data Bank (PDB) file leaving only the turns.

These turns were recorded as a PDB file format and processed with the Ramachandran algorithm to calculate and assign phi, psi, and omega angles. The resulting dataset under investigation consisted of 17225 amino acid turn-examples in total, from 11 families, with their corresponding phi, psi, and omega angles. Each of the 20 amino acid types were assigned an identification number from 1 to 20, and saved in the neural network input feature vector as a descriptor, i.e., feature.

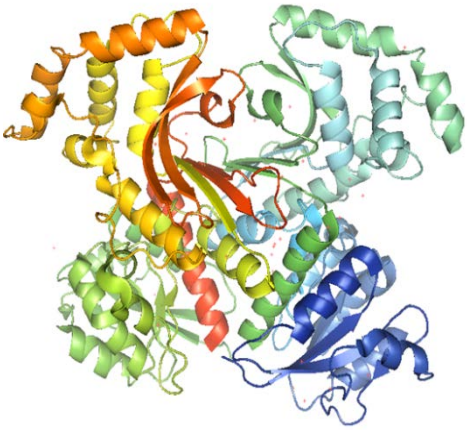

Figure 1. A schematic illustration of Homoserine Dehydrogenase enzymes where turns (loops) attach beta-sheets and alpha helices.

The present work's algorithm focuses on mapping the phi, psi, and omega angles of central amino acids in chains of 3 (triplets) in each of the 57 enzymes, i.e., amino acid chains, from the eleven families mentioned above.

The neural network input feature vector was constructed programmatically by scanning the enzyme chains for amino acid triplets, called (aai_{-1}, a_i, and aa_{i+1}) as shown in Fig.2.

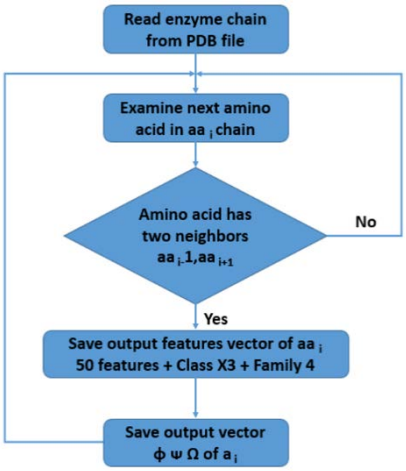

Figure 2. A Flow diagram data processing.

For each central amino acid a_i found, a feature vector was constructed containing: Fifty descriptors of a_i; class number of aa_{i-1}; class number of aa_i; class number of aa_{i+1}; family number of enzyme chain containing the triplet.

The output vector representing the central amino acid a_i is composed of the phi, psi, and omega dihedral angles of the amino acid a_i inside the enzyme turn as illustrated in Fig.3. The fifty descriptors of the triplet center amino acid a_i consisted of three groups: 15 electronic properties, 17 steric properties, and 18 hydrophobic properties which can be discussed later.

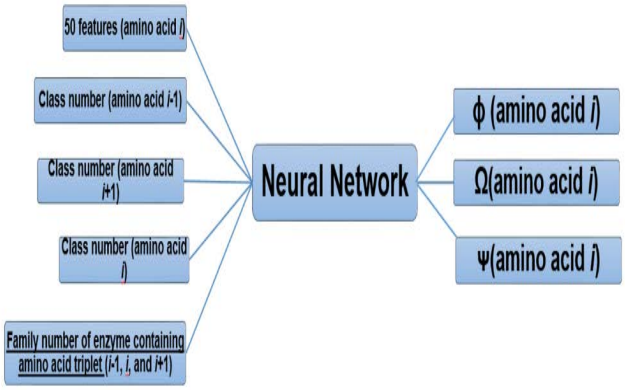

Figure 3. A schematic illustration ofneural network configuration.

2.2. Artificial Neural Network

The type of neural network (Bishop, 1995) implemented in the present work is a feed-forward Levenberg-Marquardt back propagation (Nawi *et al*., 2013), that illustrates using the gradient descent method with momentum weight and a bias learning function (Kumar and Minz, 2014). It consists of three layers of perceptrons, i.e., nodes: input; hidden; output. Each feature value in the input feature vector is connected to each of the input layer perceptron's by a multiplicative weight (Bishop, 1995).

In Fig. 4, the weights are implied parts of the arches shown. This means that the number of input layer nodes equals the length of the input feature vector, which was 53 or 54 depending on the experiment conducted.

Each hidden layer node processes the weighted sum of inputs to produce an output, which in turn feeds, via weight into each output layer node. In the present work, the number of hidden layer nodes was chosen to be equal to the number of input nodes. The output layer consists of three nodes producing three outputs, which are the angles phi, psi, and omega.

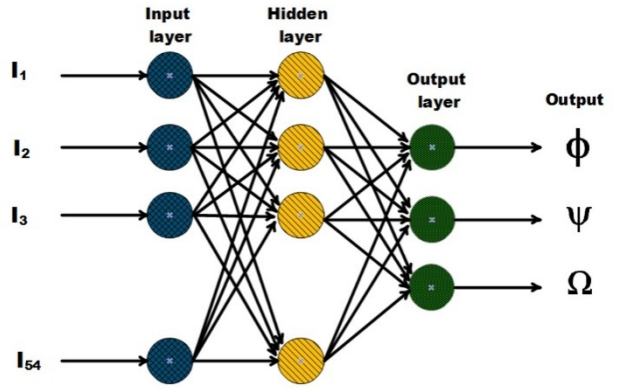

Figure 4. A schematic illustration of Feed-forward back-propagation neural network structure.

The data set was randomly divided into three parts: 60% for training the neural network; 20% for validating it and stop training before over fitting; and 20% for independent testing. The network performance was measured using the Mean Squared Error (MSE) (Kumar and Minz, 2014).

3. Results

3.1. Feature Descriptions

Physical properties of amino acids are the foremost players in building the final or even the dynamic 3D structure of a protein. Classically, interactions were classified in three groups: electronic, steric, and hydrophobic.

Fifty features have been employed in the present work classified as follows: 15, 17, and 18 features represent electronic, the steric and the hydrophobic properties, respectively. Detailed description of these features can be found in the work conducted by Mei *et al.* (Mei *et al*., 2005).

The total number of amino acid features used to predict the three angles, i.e., 3D structure is 53. These include the 50 features described above in addition to the labels of the amino acid under investigation and its two sequence (before and after) neighbors.

3.2. Enzyme Family Mapping

In order to validate the utility of the 53 selected features, a test was performed to sort out family types based on the mentioned features. Eleven family labels were used as output of the pattern recognition network. The dataset was composed of around 10 enzyme examples of each of the 11 family types. Each of the enzyme examples was processed to produce amino acid triplets with 53 features for each triplet's central amino acid, i.e., the neural network input vector. Thus, the neural network consisted of 53 inputs and 11 outputs.

The numbers of hidden layer nudes were 10 and the dataset was divided into three parts: 70% for training; 15 % for validation and prevention of over training; and 15 % for independent performance testing.

Performance was evaluated using two measures: Mean Square Error (MSE) between outputs and targets and the confusion matrix percentage of correct classification, these were 0.078 and 76.4 %, respectively.

3.3. Intra-Family Structure Prediction

The original dataset was divided by family type and inside each family, neural networks were trained to predict the dihedral angles phi, psi, and omega of the central amino acid in the triplets based on 53 descriptors. Structural elements were predicted for each family in a separate training set.

Table 1 shows the regression coefficient (R) as a measure of alignment validation. In general, EC 1.1.1.X families where X = 1, 2, 8, 9, 10, 14, 17, and 18 shows an R-value above 0.5. EC 1.1.1.X with X=10 shows the highest value of 0.8 where the poorest value was recorded for X=21. The training regression is shown in Fig. 5 for the best Enzyme family.

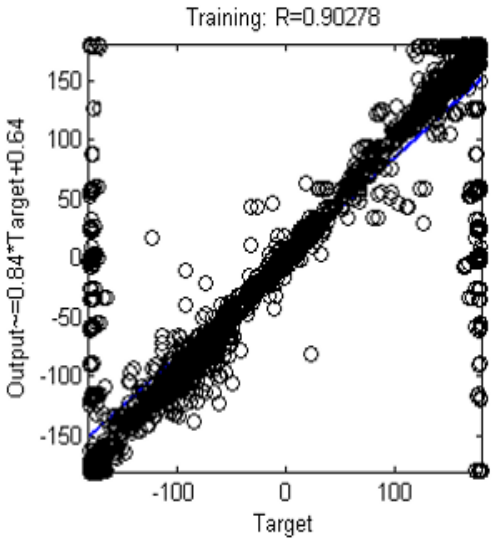

Figure 5. Training regression result for EC 1.1.1.10 with 53 features as input and 53 hidden layer nodes

Table. 1. Final results of neural network training before feature extraction (sorted by performance)

Enzyme's family	R-value	Number of iterations	Training time (min)
EC 1.1.1.10	0.84147	131	7:22
EC 1.1.1.9	0.79310	124	6:20
EC 1.1.1 14	0.75090	132	6:20
EC 1.1.1.1	0.74688	196	14:48
EC 1.1.1.2	0.69972	95	4:52
EC 1.1.1.8	0.55979	74	3:33
EC 1.1.1.18	0.52505	89	6:34
All Families	0.50942	131	25:11
EC 1.1.1.17	0.50326	74	3:44
EC 1.1.1.3	0.48436	73	3:32
EC 1.1.1.22	0.35053	52	2:30
EC 1.1.1.21	0.31822	69	2:53

3.3.1. Inter-Family Structure Prediction

Based on the intra-family result, additional training was performed where the family labels were added to the input features (making 54 features in total), and the amino acid triplets' structures were predicted across all families.

Results are summarized in row number eight in Table 1 that shows low performance, which may indicate the demand to extend the size of the input feature vector. In addition, the structure of the central element of the amino acid triplets varies with family type.

3.3.2. Feature Selection

Feature selection can be defined as a process of feature-selection, or an applicant subset of features. In order to set the evaluation criteria, few feature subsets are used. The present study enables a promising method that predicts the dihedral angles of turn structures by the means of feed-forward Levenberg-Marquardt neural network. Feed-Forward selection enable finding weaker subset of features, due to the face that weaker features are not assessed while subset selection (Kumar and Minz, 2014).

Several neural networks have been trained to predict the structure of the central element of amino acid triplets across variable number of features and hidden layer nodes. All of the networks employ an input vector length of 54 features as in the intra family structure prediction described earlier.

Results are summarized in Table 2 and show no significant configuration that outperforms the base case of 54 with 50 hidden layer nodes. As and additional effort, the training parameters were modified for two cases to test whether the results could be improved.

The number of training epochs was raised from 1000 to 2000, and the failure checks were changed from 66 to 2000. The results were R = 0.33062 for 32 features and 50 hidden layer nodes, R = 0.47658 for 43 features and 50 hidden layer nodes.

In the first case, the performance (relative to 54 features and 50 nodes) decreased probably due to over fitting. In the second case, the performance is slightly worse than the base case, probably due to the elimination of 10 features.

Table. 2. Network performance with varying feature node and number (Shown is the total R-value)

	No. of Features					
No. of Nodes	5	16	22	32	43	54
25	0.46448	0.46619	0.46643	0.46893	0.46488	0.49363
50	0.47322	0.46858	0.47634	0.46514	0.47146	0.49657
100	0.47517	0.47678	0.47509	0.46804	0.28371	0.50777
200	0.42271	0.25977	0.46628	0.31304	0.44277	0.51903

The results of inter-family predictions in Table 2 indicate dependence of the amino acid triplet structures on the family type of which they belong. Thus, the potential of feature reduction was investigated from the EC 1.1.1.10 family only, using 53 features (with family label removed) and 50 hidden layer nodes. The R-value for this case was 0.80192. The objective was to reduce the number of features, without significantly lowering the R-value.

In order to reduce the number of features, the sum of absolute perceptron weights for each of the 53 inputs was computed. A threshold of 15 was chosen (as shown in Fig. 6) to discard all features with an absolute sum of feature weights below this threshold.

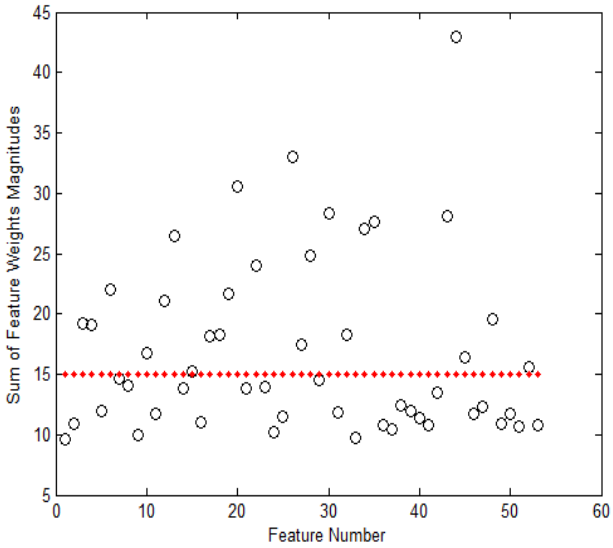

Figure 6. Feature extraction based on the sum of absolute perceptron weights for each of the 53 inputs where 15 was used as a threshold

The number of discarded, i.e., minor features, was 29, which is about 54.7% of the original 53 features. The R-value was 0.69366, which can be considered good because it represents a performance degradation of only about 13.5% when compared to the R value of 0.80192 for the 53 feature case.

The major features that remained are listed in the input vector:

[3, 4, 6, 10, 12, 13, 15, 17, 18, 19, 20, 22, 26, 27, 28, 30, 32, 34, 35, 43, 44, 45, 48, 52]

These major features are summarized descriptively as follows: 9 features were found in the hydrophobic properties including:

- solvation free energy
- Melting point
- Number of full nonbonding orbitals
- Retention coefficient in HPLC, *pH* 2.1
- Retention coefficient at *pH* 2,
- R_f for 1-N-(4-nitrobenzofurazono)-amino acids in ethyl acetate/pyridine/water
- Hydration potential or free energy of transfer from vapor phase to water
- Log D
- Partition coefficient at pH 7.1 for acetylamide derivatives of amino acids in octanol/water
- $dG = ¼RT \ln f$, where f = ¼fraction buried/accessible amino acids.

Other 10 features appeared in steric properties:

- Average volume of buried residue
- Residue accessible surface area in tri-peptide
- Normalized van der Waals volume
- Average accessible surface area
- Distance between C_α and centroid of side chain
- Side-chain angle
- Radius of gyration of side chain
- van der Waals parameter epsilon
- value of θ *(i)*
- Substituent van der Waals volume.

And four features originated from electronic properties:

- Negative charge
- Polarity
- Net charge
- Electron-ion interaction potential values

Furthermore, one of the major features was the type of central amino acid in the sub group (a_i).

4. Conclusions

The present study gives a method to predict the dihedral angles of turn structures by feed-forward Levenberg-Marquardt neural network. The datasets for training and testing the network are PDBs of eleven different families of enzymes from Expert Protein Analysis System (ExPASy) and protein data bank. Secondary structures consisting of α-helices and β-strands were removed from the PDB file leaving only the turns.

A feature vector containing around 53 parameters was constructed for each central amino acid of amino acid triplets. This vector is used as input for neural network.

Ninety-seven enzyme families were selected and preconditioned to be an input vector for a feed forward back propagation neural network. The dihedral angles of only the turns in the 3D structures were predicted after training.

The structural features of dissected triplets show significant influence on family type. R-values for the inter-family data set as well as feature selection were not satisfying. In contrast, the R-values of about 0.8 were achieved in the case of intra-family prediction.

In addition, it is believed that the structural features of dissected triplets show significant influence on family type. About 55 % of features can be eliminated with relatively less negative influence of 13% on the R value. The present paper can provide promising useful prediction method that can advance the computational methods in bioinformatics, especially about the prediction of 3D protein structure as a function of enzyme family type and amino acid sequences.

To the best of our knowledge, many researchers have established various methods to predict protein structure. However, the intra-family prediction probability is higher when only one type of analysis based on the dihedral angles of turn structures of enzyme families is used. Therefore, biochemical experiments can be used for validation of the proposed prediction method that will enable reliable and shorter time experiments in the field of bioinformatics.

Conflict of Interest Statement (COI)

The authors declare that they have no conflict of interest.

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Molecular Cloning and in *Silico* Analysis of *rps7* Gene from the *Lactuca sativa*

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Abstract

The *rps7* genes encode 30S ribosomal protein S7 that bind directly to the parts of the 3'end of 16S ribosomal RNA. The *rps7* gene is one of the most important plastidial genes that have not been studied so far in lettuce (*Lactuca sativa*). The molecular analysis of *rps7* gene provides an opportunity to make phylogenetic studies and a conserved homologous recombination site for designing chloroplastic expression vectors with high-efficiency lettuce cultivars and its related species. Here, we have cloned plastid *rps7* gene from lettuce. The full length of lettuce *rps7* gene is 2579 bp, composed of the 289 bp promoter region, an Exon 468 bp long encoding 155 amino acids. The *rps7* gene contains a conserved domain (1-155). The promoter region analysis showed that the cloned fragment contains motifs, like TATA box, CAAT-box, MBS, AAGAA-motif and GA-motif. The alignment analysis *rps7* showed the high homology with identity of 96% compared with *Asteraceae* family at the nucleotide level, while the amino acid sequence of the gene showed a high similarity value of 98.1-90% compared with hairy sunflower, wormwood, ragwort and soybean. The phylogenetic analysis result shows the *rps7* gene from dicots is close to *rps7* gene from lettuce varieties.

Keywords: Plastid Genome, Lettuce, *rps7* Gene, PCR

1. Introduction

Chloroplasts are heterogeneous plastidial organelles responsible for many metabolic processes such as photosynthesis, synthesis of amino acids, fatty acids and secondary metabolites (Marín *et al*., 2007). Chloroplasts contain circular double-stranded DNA with 76-217kbp long includes approximately 120-130 genes present in 1,000–10,000 copies per cell, and maternally inherited in the most angiosperm-plant species. Although they have prokaryotic origin distinct from the nucleus, their transcription factors with prokaryotic characteristics can be matched with the transcription factors of eukaryotic mRNAs. Furthermore, they are polyploid in higher plants and they have an extremely conserved organization (Raubeson and Jansen, 2005), (Hinsinger and Strijk, 2015) and (Zhang and Gao, 2016). The proteins specifically Multi-subunit functional protein complexes that are involved in photosynthesis are encoded by plastidial genome and directly synthesized within the chloroplast (Daniell *et al.*, 2016). Because of its abundance in plant cells and ease of sequencing, chloroplast DNA (cpDNA) has been widely utilized in studies of plant taxonomy and evolution (Kress and Erickson, 2007) and (Taberlet *et al*., 2007).

The *Lactuca sativa* chloroplast genome (DQ383816) with 152772 bp-long contain an inverted repeats (IRs) (25034 bp) that are separated by a large (84105bp) and small single-copy (18599bp) (LSC and SSC) regions. Moreover, the cpDNA from *L.sativa* contain several genes including encoding genes of tRNAs, rRNAs, RNA polymers, ribosomal proteins, ATP synthesis, cytochrome b6/f, NADH dehydrogenases. In higher plants, a pair of *rps7* genes encoded ribosomal protein S7 protein from the small ribosomal subunit was located in Inverted Repeats (IRs) regions of the plastid genome. Therefore, *rps7* genes can be used as a homologous recombination site to achieve high yield expression level of a gene of interest. Following, a gene of *rps7* that is located in the IRa, is transcribed in the clockwise direction and the other that is located in the IRb, is transcribed in the counter clockwise direction (Timme *et al*., 2007). In *Escherichia coli*, S7 is known as the protein that binds directly to the parts of the 3'end of 16S ribosomal RNA. It belongs to a family of ribosomal proteins, which have been grouped on the basis of sequence similarities (Klussmann *et al*., 1993) and (Ignatovich *et al*., 1995). This entry represents the S7

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structural domain, which consists of a bundle of six helices and an extended beta hairpin between helices 3 and 4 with two or more RNA-binding sites on its surface (Wimberly *et al*., 1997). This entry also represents 30S ribosomal proteins S7 (bacterial, archaeal, plastid, mitochondrial), and eukaryotic 40S ribosomal proteins S5 (cytoplasmic). The 30S ribosomal protein S7 make an interaction between ribosomal proteins of S9 and S11. It is also one of the primary rRNA binding proteins that bind directly to 16S rRNA, where it nucleates assembly of the head domain of the 30S subunit S7 is located at the subunit interface close to the decoding center shown to contact mRNA (Robert and Brakier-Gingras, 2001). It has also been shown to contact tRNA in both the P and E sites, it probably blocks the exit of the E site tRNA.

At higher taxonomic levels (family level), protein-coding regions and conserved sequences of the chloroplast genome can be used to phylogenetic analysis and domestication studies (Jansen *et al*., 2007). Since, the *rps7* gene is one of the most important genes of chloroplast genome with a conserved structure, the molecular analysis of this gene can be used for phylogenetic studies. Understanding the genetic relationships between crops and their close relatives would provide an attempt to introduce specific advantageous traits into the related crops.

2. Materials and Methods

2.1. Plant material and DNA Extraction

Lettuce (L.*sativa.var*. *Salinas*) seeds were planted in plastic trays, and let to grow to 2-3 leaf stage before extracting their DNA. Total genomic DNA was extracted from fresh leaves using a modified CTAB method (Doyle, 1987; Yang, Li & Li, 2014). Quality, quantity and concentration of the extracted DNA were determined by 0.8% agarose gel electrophoresis.

2.2. Designing of Primers

To obtain the complete sequence of the lettuce *rps7* gene a pair of specific primer was designed by using Primer3 online software. Sense and anti-sense primers for this fragment were (F: 5'-AGTTTTCATTCTGTACATGCCAG -3') (R: 5'-CTCACTAAGCCGGGATCACT -3').

2.3. PCR Amplification and Bacterial Transformation

Total genomic DNA of lettuce was used as a template for amplification of the target fragment in the concentration of 5 ng/µl for the fragment. The amplification profile was 94°C for 5min, followed by 35 cycles of 94°C for 30 Sec, 50°C for 40 Sec, and 72°C for 1min and 20 Sec, and a final extension step at 72°C for 10min. The amplified DNA product was purified by using a gel extraction kit from Bioneer Company. The ligation reaction of the related fragment with Vivantis company *p*TG19-T vector was done through T/A cloning by using *T4* DNA ligase enzyme (produced by Termo Company). In order to transform, first *E.coli* strain DH5α bacterial competent cells were prepared by TSS method, then thermal shock method was used for transformation of competent cells. Transformants were selected on white-blue test medium containing antibiotic ampicillin, IPTG and X-gal.

Colony PCR was performed to confirm the presence of the cloned fragment in the white colonies bacteria. The plasmid DNA was extracted from the liquid culture of PCR positive colonies using a plasmid extraction kit from Bioneer Company. Finally, the recombinant plasmids DNA was reconfirmed with *EcoR*I (produced by Termo Company) digestion (because the restriction site of this enzyme is located on the *p*TG19-T) and was sent for sequencing to Bioneer Co.

2.4. Bioinformatics Studies

Alignment of plant *rps7* At first, DNA-to-protein translation of DNA sequences was performed using the EXPASY translate tool (http://au.expasy.org/tools/dna.html) then the amino acid sequence of the lettuce *rps7* gene was compared with other *rps7* genes by ClustalW2 (www.ebi.ac.uk/Tools/msa/clustalw2/) software. Sequence analysis of rps7 promoter to find regulatory elements in promoter sequences, was done by PLANTCARE (http://Bioinformatics.psb.ugent.be/webtols/plantcare/html) software. Domain detection was done for finding all known motifs by using the EXPASY Motif Scan tool (http://myhits.isb-sib.ch/cgi-bin/motif_scan).The phylogenetic analysis was conducted by NCBI.

3. Result and Discussion

Using primers generated from the plastid DNA sequence *rps7* of IRa, DNA fragment of 2629 bp was synthesized by PCR from lettuce plastid DNA as a template (Figure 1). This fragment was cloned into *p*TG19-T. (Fragment length=2579 bp and *p*TG19-T length=2880 bp).

The full length of lettuce *rps7*gene (L.*rps7*) is 2579 bp, composed of the 289 bp promoter region, an exon and 1822 bp3'terminus. Exon is 468 bp long, beginning with nucleotide 1, extending to nucleotide 468 encoding 155 amino acids (Figure 2).

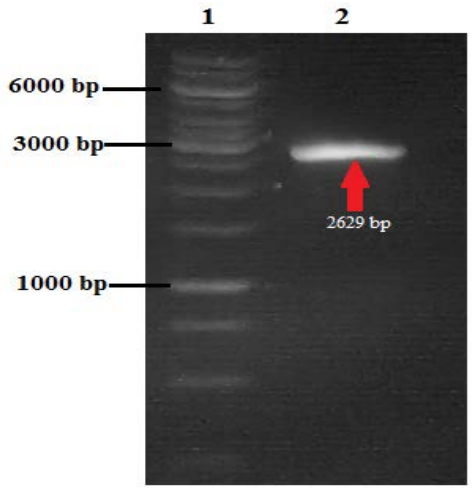

Figure 1. 1: DNA marker 2: PCR product

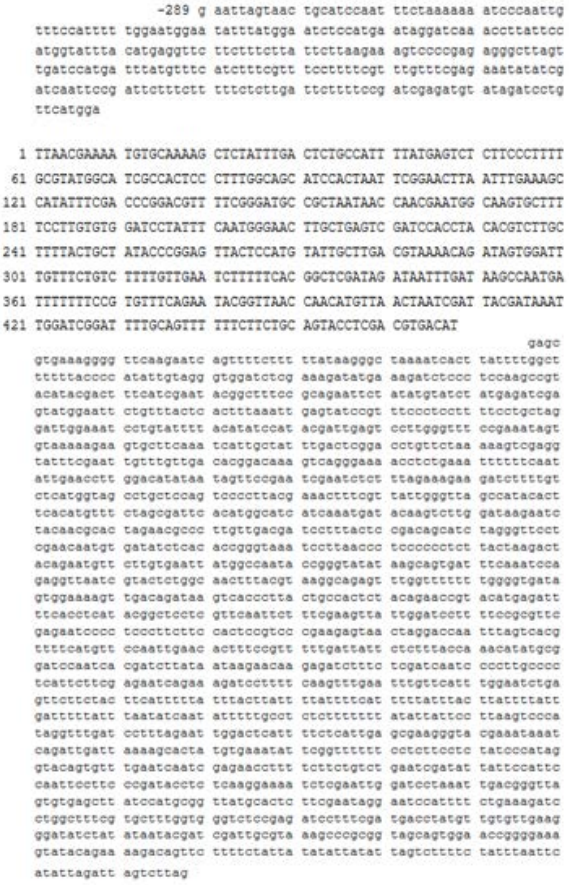

Figure 2. The nucleotide sequence of the *rps7* gene. From -1 to -289 is Promoter region. Exon is 468 bp long beginning with nucleotide 1, extending to nucleotide 468

An inserted fragment in the vector *p*TG19-T was further corroborated by PCR and digestion by restriction enzymes of *Eco*RI (Figure 3).

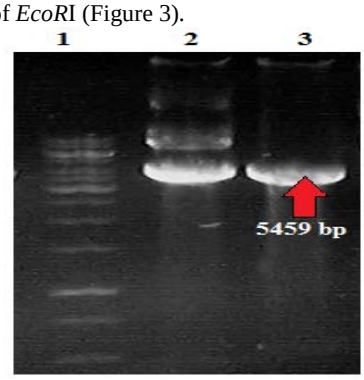

Figure 3. Gel agarose description of digested fragments. The extracted plasmids were digested by enzyme *Eco*RI 1: DNA marker 2: uncut 3: cut

3.1. Alignment of Amino Acid of rps7

DNA-to-protein translation of DNA sequences was performed using the EXPASY translate tool using the standard genetic code. The deduced amino acid sequence of the lettuce *rps7* gene was compared with seven other *rps7* genes (Figure 4). The amino acid sequence showed a high level of identity with the genes of *Lasthenia burkei*, *Helianthus hirsutus* and *Artemisia montana* (98.1%), *Jacobaea vulgaris* (96.8%), *Elaeis oleifera* (95.5%). There was 50% identity with the genes of *Spinacia oleracea*, *Solanum lycopersicum* and *Lotus japonicas*. The identity dropped to 56.9%, 56.1%, 54.8% and 54.8% with *Microcoleus sp*, *Raphidiopsis brookii*, *Nostoc sp* and *Anabaena variabilis*, respectively. Nevertheless, 74 amino acids were conserved in all eight of the considered *rps7* genes, but most of the amino acids of this protein are conserved in plants. These conserved amino acids are probably involved in the important biological functions of polypeptides.

The nucleotide sequence of *rps7* was also compared with other *rps7* genes. This sequence showed a high level of identity with the *rps7* gene of other plants especially with *Asteraceae* family. This observation is consistent with the view that the sequence of the plastid genome is more conserved than the same sequence of the nuclear genome. This may also allow the use of the *rps7* gene as a homologous recombination site of different plants for plastid transformation.

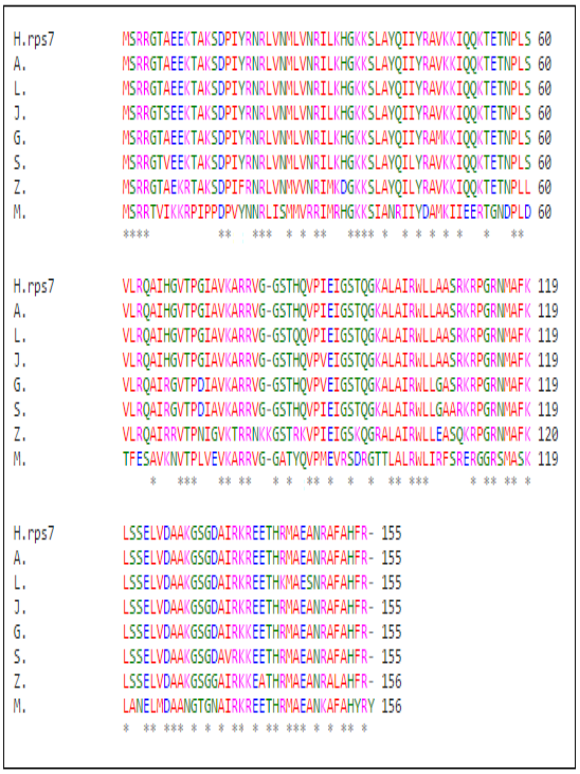

Figure 4. Comparison between the deduced amino acid sequence of *Lactuca sativa* plastid *rps7* (L.*rps7*) and the corresponding sequences from *Helianthus hirsutus* (H.*rps7*), *Artemisia montana* (A.*rps7*), *Jacobaea vulgaris* (J.*rps7*), *Glycine max* (G.*rps7*), *Spinacia oleracea* (S.*rps7*), *Zea maize* (Z.*rps7*) and *Microcoleus sp* (M.*rps7*) Identical amino acids are marked by asterisks in all 8 sequences

3.2. Sequence Analysis of rps7 Promoter

To find regulatory elements in promoter sequences, the PLANTCARE software was applied. Analysis of the sequences of the promoter fragment revealed several kinds of specific promoter motifs in 289 bp of these cloned promoter fragments as shown in (Figure 5) and description of each one shown in Table 1.

The CAAT-box motif with common cis-acting element in the promoter and enhancer regions is often present at -80 to-150 bp upstream of TIS (Transcription Initiation Site) and may operate cooperatively with other putative conserved motifs (Gelinas, 1985). However, no unifying expression pattern of plant genes containing putative CCAAT elements (Rieping and Schoffl, 1992).

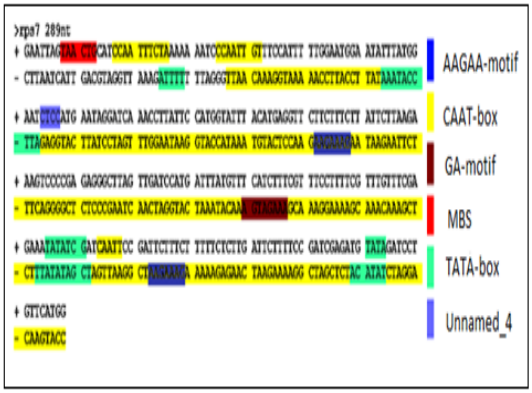

Figure 5. Sequence analysis of *rps7* promoter fragments

Table 1. Regulatory elements in *rps7* promoter sequence of lettuce

Site Name	Position-Strand	Sequence	Function
CAAT-box	38-	CAAT	Common cis-acting element in promoter and enhancer regions
	224+,18+, 36+,37-	CAATT	
	17+,35+	CAAAT	
GA-motif	180-	AAAGATGAA	Part of a light responsive element
MBS	8+	TAACTG	myB binding site involved in drought inducibility
TATA-box	214+	ATATAT	Core promoter element around -30 of transcription start
	215+,	TATA	
	271+ 213-	tcTATATAtt	
	25-	TTTTA	
	62-	tcTATAAAta	
	269-	TATACA	
AAGAA-motif	558-	GAAAGAA	
Unnamed_4	74+	CTCC	

Moreover, multiple copies of the genes coding for the subunits of the CCAAT, -binding protein exists in *Arabidopsis thaliana, Glycine max and Hordeum vulgare*, suggesting the potential for multiple alternative forms of these complexes in plants (Shirsat *et al*., 1989) and (Edwards *et al*., 1998). The first element described as regulating this process was a classical TATA box, TATA (A/T) A, located -25 to -30 base positions upstream of TIS (Davison *et al*., 1983). However, subsequent studies suggested that AT-rich sequences completely unrelated to the TATA-box stimulate transcription with equal or increased efficiency (Singer *et al*., 1990). Furthermore, although the first step of transcription initiation is highly specific, TFIID also binds with high affinity to several TATA elements that do not match the consensus sequence and is active in promoting transcription in vitro from these elements (Hahn *et al*., 1989). In eukaryotic promoters, between 10 and 20% of all genes (Gershenzon and Ioshikhes, 2005) contain a TATA box (sequence ATATAT, TATACA, TATAA, TTTTA, TATA, tcTATATAtt, tcTATAAAta), which provides for a TATA binding protein and assists the formation of the RNA polymerase transcriptional complex (Smale and Kadonaga, 2003). The TATA box typically lies very close to the transcription initiation site (often within 50 bases), and tends to be surrounded by GC rich sequences. The GA-motif is part of a light, responsive element that is similar to this motif in *Helianthus annuus* - motif (Eghtedary, 2014). MBS (myB binding site) has also been localized in the upstream promoter region that involved in drought and it is similar to this motif in *Arabidopsis thaliana* (Van Moerkercke *et al*., 2011). AAGAA-motif element is present in 19.23% of the genes. This element contains sequences GAAAGAA and GTAAAGAAA, and its function is unknown (Karimzadeh *et al*., 2013). We suggest designing the 5'UTR in genetic engineering and gene transfer issues to make the plants more stress tolerant, use above cis-elements especially CAAT-box, TATA-box and MBS.

3.3. Domain Detection

Domain detection was done for finding all known motifs that occur in a sequence using the EXPASY Motif Scan tool. As a result, the domain of 30S ribosomal protein S7 (1-155) whit N-score=30.261 and E-value= 1.2e-23 shown in (Figure 6).

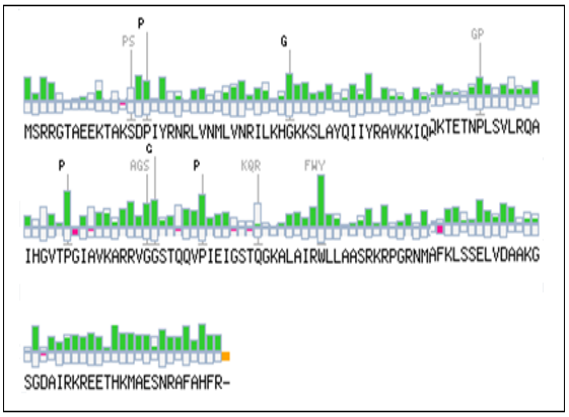

Figure 6. The green parts of the figure represent the amino acid of domains is in its proper place and red parts show the amino acid is not in its proper place compared with a protein domain which can be compared

3.4. Relationship Analyses of the rps7 Gene

The phylogenetic analysis was conducted by NCBI to investigate the closely related species to L.*sativa*. The resulted phylogenetic tree was divided into two clusters. The first cluster contains species of monocots and the second cluster includes species of dicotyledons. According to the results of the phylogenetic tree, the *rps7* gene sequence of dicotyledonous species is close to *rps7* gene sequence of lettuce varieties (Figure 7).

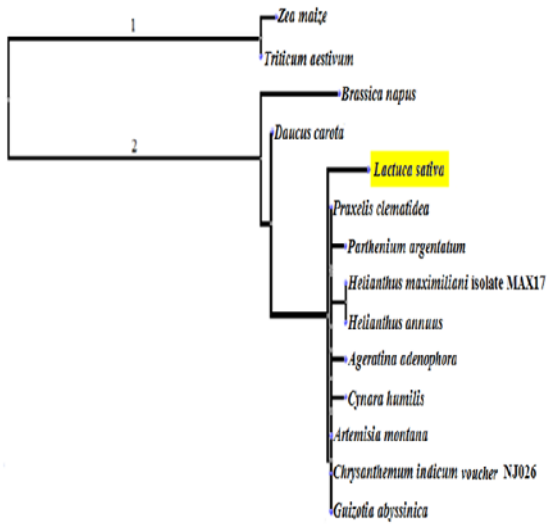

Figure 7. Phylogenetic relationship of *rps7* from *Lactuca sativa* and the other close species

4. Conclusion

The aim of the present work was to clone and characterize *rps7* gene from *Lactuca sativa* plastid which has not been studied so far in lettuce. The PCR fragment was cloned into *p*TG19-T cloning vector. The fragment was validated by *Eco*RI restriction digestion analysis and verified by sequencing. The alignment analysis results were analyzed showed the high homology with identity of 96% compared with *rps7* gene from lettuce. The genetic and phylogenetic studies of this gene can be used to identify varieties and in the conservation of breeding resources. Success in breeding is determined by genetic compatibility and chloroplast genomes serve as a valuable tool for identifying plants that are likely to be closely related and, therefore, genetically compatible. On the other hand, the *rps7* gene can be used as a secure homologous recombination site for plastid transformation with high-efficiency for lettuce cultivars and the other close species as well.

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Effect of UV-B Radiation on Chromosomal Organisation and Biochemical Constituents of *Coriandrum sativum* L.

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Abstract

Stratospheric ozone depletion due to pollution has long been recognised as a threat to human health as well as to the earth's ecosystem. UV-B radiation being a part of solar electromagnetic radiations reaches earth's surface at an elevated level due to ozone depletion thereby imparting its ill impacts on flora and fauna. Hence, keeping UV-B as an important key of environmental factors inducing stress and disturbance on biodiversity the present experimental work has been designed to study the effect of UV-B on chromosomal organisation and biochemical contents of *Coriandrum sativum* L. Four sets have been maintained *viz.* set A for control, set B for 20 minutes treatment, set C for 40 minutes and set D for 60 minutes. All sets, excluding A, were irradiated with supplemental UV-B radiation along with visible lights for 1st, 2nd and 3rd day treatment after seed germination. For cytological study, irradiated germinated seeds of each set (B, C and D) were fixed in carnoy's fixative along with control (set A). For biochemical study few germinated irradiated seeds of each set (A, B, C and D) were transplanted in field for further growth and development. It was found that lower doses are stimulatory in its action while as the treatment time along with duration was increased, the rate of Mitotic Index (MI %) were decreased and Total Abnormality Percentage (TAB %) was increased in all the treated sets as compared to control set. Regarding biochemical constituents, the proline content was increased while photosynthetic pigments (chlorophyll a, chlorophyll b and carotenoid) and total carbohydrate contents were declined at higher treatment doses. Hence, it was concluded that low levels of UV-B exposures are not inhibitory in its action and promote metabolic processes on the way in contrary high levels of elevated UV-B radiation are genotoxic and causes cell disruptions by inducing chromosomal aberrations increasing TAB (%), declined MI (%), decreased photosynthetic pigments (chlorophyll a, chlorophyll b and carotenoid) and carbohydrates while in response to stress, *Coriandrum* adapts protective mechanism thereby elevated proline accumulation.

Key words- UV-B radiation, Mitotic Index (MI %), Total Abnormality Percentage (TAB %), Proline, Photosynthetic pigments, Carbohydrate, *Coriandrum sativum* L.

1. Introduction

Biodiversity in plants with its variety and variability contains enormous potential in meeting humans growing economic needs. Several hundreds of species have served as bio-resources of great potential, during the course of time as human civilization grows. Human intervention through centuries for food, fibre, shelter and medicine has altered the dynamic relationship among the various ecosystems leading to disturbed natures functioning. Ozone depletion is an outcome of modern scientific and technological advances that results into more penetration of Ultra-Violet (UV) radiation on earth's surface thereby imparting ill-effects on biological components. UV radiation is part of the sun's electromagnetic radiation, classified into UV-A (320-400 nm), UV-B (280-320 nm) and UV-C (200-280 nm). UV-B is of particular interest because this wavelength represents near about 1.5% of the total spectrum but can induce a variety of damaging effects. As plants are the primary producers in an ecosystem and form the basis of bioactive systems hence they are more threatened to adverse effects of radiation. The sessile lifestyle of plants particularly necessitates the evolution of a number of strategies for adaptation to an ever-changing environment. Of utmost importance is light, which is not only a source of energy but also provides informational signals concerning the surrounding natural setting, influencing plant growth and development. UV-B can cause severe deleterious effects in biological organisms, despite representing only a small amount of total solar radiations. UV-B can adversely react with many biological molecules including amino acids, nucleic acids, proteins, lipids and elicits stress responses at molecular, cellular and whole organism levels. Garinis *et al.* (2005) stated that UV-B can damage DNA by creating cyclobutane pyrimidine dimers and pyrimidine (6-4) pyrimidine dimer, which can lead to point and break mutations if not correctly repaired. Certain responses were elicited by plants including inhibition of hypocotyls

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elongation and root growth, cotyledon opening stomatal closure and anatomical changes associated with UV-B protection. Different species have different responses to the level of UV-B irradiation (Matthew *et al.*, 1996; Skorska 1996 a, b). The changes in plants morphology induced by UV-B may affect competition for light (Barnes *et al*., 1988). The negative effect of UV-B radiation results in deformed morphological parameters. Exposure to UV-B decreased plant height, leaf area and plant dry weight increased auxiliary branching and leaf curling (Dai *et al*., 1995; Greenberg *et al*., 1997; Furness *et al.*, 1999). Dai *et al.*, (1995) reported that after a few weeks of UV-B exposure, leaf area and plant dry weight of rice were significantly reduced. High levels of UV-B clearly decreased the relative growth rate and nitrogen productivity, as leaf area ratio, leaf area productivity and leaf nitrogen productivity were all decreased (Zuk-Golaszewska *et al*., 2003). Research studies had traditionally focussed on staple crops while little attention has been given to minor crops. The limited information available on many important and frequently basic aspects of underutilized crops hinders their development and their sustainable conservation.

Coriandrum sativum L. commonly called as Coriander is an important spice crop of Apiaceae possessing 2n=22 chromosomes having diverse economical uses. It has been widely used as a culinary ingredient as well as traditional remedies for the treatment of different disorders, like hyperglyceamia, antispasmodic, carminative, stimulant, cytotoxic, lipolytic, fungicidal and stomachic compound. Coriander also possesses hypolipidemic, antibacterial, antimutagenic activity, insecticidal and aflatoxin controlling effects primarily due to its essential bioactive compounds.

Considering the aforesaid features, in the present study coriander is selected as an experimental model as it is easily available throughout the year. Hence, the present research work has been designed to screen out the effect of UV-B radiation on biochemical constituents and chromosomal organisation of coriander. Chromosomal study was done in root meristems of coriander as these are the first to emerge and interact with environment. After an extensive review of literature, it was found that the present study is the first one on coriander of its kind, which will elicit further light on this subject.

2. Material and Methods

2.1. Seed Procurement

Seeds of Coriander were collected from research institute *viz.*, CRSS, Jagudan, Gujarat, *variety* CO-2. Seeds were consistently selected and proper washing was done for 10 minutes with distilled water and 0.1% $HgCl_2$ was utilized for sterilization.

2.2. Experimental Design

Fresh Coriander seeds were pre-soaked in distilled water for 12 hours and kept in seed germinator at 25 ± 20^0C with humidity 60-80% in sterilized petriplates with wet whatmann filter paper. Whatmann filter papers were regularly allowed to change and distilled water was sprinkled periodically. Four sets were prepared, i.e., Set A for control, Set B for 20 minute, Set C for 40 minute and Set D for 60 minute. The experiment was conducted by keeping nine replicates.

2.3. UV-B Treatment

Sets B, C and D having early roots of length between 5mm to 25mm were irradiated with fluorescent UV-B (280-320 nm) lamps along with visible light. Firstly, 9 replicates of each set were irradiated for1 day of time duration 20, 40, 60 minute along with supplementation of visible light. Out of which three replicates from each set were removed off from UV-B chamber for fixation. On second day next 6 replicates of each set were again irradiated for respective time duration and three replicates were removed off for fixations. For remaining three replicate out of 9 same procedures was followed for each set. Set A was remained untreated as standard. Radiation was started in the morning on each day.

2.4. Fixation

After one hour of recovery, all the irradiated germinated seeds of set B, C, D along with control set A were fixed in Carnoy's fixative in their labelled bottles for cytological study. After 24 hours of fixation, Carnoy's fixative was decanted off and sets were transferred into bottles containing only 90% alcohol.

2.5. Mitotic Preparation

For cytological study, squash technique was applied. Staining was done with 2% acetocarmine for half hours. Slides were prepared and cells were observed and snapped under Nikon Research Electron Microscope using Olympus PCTV Vision Software. Nearly 10 microscopic field views were recorded from each slide. Data were scored from 3 roots of each replicates.

2.6. Formula Used for Scoring of Data

To calculate Mitotic Index (MI %) and Total Abnormality Percentage (TAB %), the following formulas were followed **:-**

Mitotic index (MI) % = (Total number of dividing cells/Total number of observed cells)*100

Total abnormality percentage (TAB) % = (Total number of abnormal cells/Total number of observed cells)*100

2.7. Biochemical Analysis

2.7.1. Estimation of Proline

Total proline was estimated by using Bates *et al.* (1973) method.

2.7.2. Carbohydrate Estimation

Determination of total carbohydrate was done by using Hedge and Hofreiter (1962) method and absorption was taken at 630 nm.

2.7.3. Determination of Photosynthetic Pigments

Chlorophyll a, b, and carotenoids were extracted from fresh leaves of coriander with 80% acetone and determined according to Lichtenthaler method (1987).

2.8. Statistical Analysis

The data obtained were analysed using statistical software, SPSS 16 and means were compared using Duncan's Multiple Range Test (DMRT) ($P \leq 0.05$). All the results were expressed in form of Mean ± Standard Error. The graph was plotted by using Sigmaplot 10.00 software.

3. Results

3.1. Cytological Observations

Mitosis was found to be normal in the control sets and showed regular arrangements of chromosomes at metaphase (2n=22) and having equal separation (22:22) at anaphase. However, various chromosomal abnormalities were recorded in root meristems of sets B, C, D raised for supplemental UV-B treatments.

3.1.1. Effect on Mitotic Index (MI)

The collected data infer that lower doses of UV-B are less significant at 1st day treatment while imposes impairment in regular cell division at 3rd day treatment. But higher doses of UV-B treatment caused a strong mitodepressive effect on meristematic cells of root tips of Coriander documented in table 1. In control set, MI was 12.64 ± 0.14^{a} however, the rate of MI (%) declined as the treatment duration increased. On 1st day at 20 min treatment MI was 11.42 ± 0.26^{b} which was reduced to 10.41 ± 0.13^{b} on 3rd day treatment that envisaged less effect of lower doses of UV-B treatment. Meanwhile, at 60 min treatment MI was recorded to be 8.19 ± 0.33^{d} on 1st day compared to 20 min it shows steep declined and on 3rd day MI was steadily declined to 6.75 ± 0.11^{d}. Hence, from above results, it was clearly inferred that higher doses of UV-B treatment are mitodepressive. The pattern of declined MI along with UV-B treatment was shown in Figure 2 and Table 2.

Table 1. Total account of Mitotic Index and Total abnormality percentage after UV-B radiations on root meristems of *Coriandrum sativum* L.

TREATMENT (UV-B)*	DOSES (Minutes)	MI (%)** (Mean ± S.E.)	TAB (%) *** (Mean ± S.E.)
1st DAY	Control	12.64 ± 0.19^{a}	-
	20	11.42 ± 0.26^{b}	2.53 ± 0.14^{c}
	40	10.12 ± 0.24^{c}	3.31 ± 0.26^{ab}
	60	8.19 ± 0.34^{d}	4.16 ± 0.32^{a}
2nd DAY	Control	12.64 ± 0.14^{a}	-
	20	11.21 ± 0.38^{b}	3.54 ± 0.12^{c}
	40	9.13 ± 0.38^{c}	4.84 ± 0.31^{b}
	60	7.74 ± 0.02^{d}	6.34 ± 0.12^{a}
3rd DAY	Control	12.64±0.14a	-
	20	10.41 ± 0.13^{b}	4.64 ± 0.33^{c}
	40	8.43 ± 0.11^{c}	6.70 ± 0.26^{b}
	60	6.75 ± 0.11^{d}	8.58 ± 0.37^{a}

Abbreviations: UV-B- Ultraviolet B radiation, MI (%) **- Mitotic Index, TAB (%) ***- Total abnormality percentage. Means are followed by lowercase letter is statistically significant at $p<0.05$.

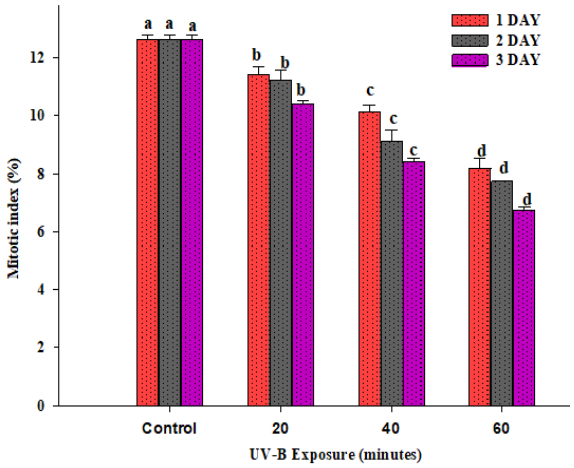

Figure 2. Comparative trend of MI on different doses of UV-B treatment on root meristems of *Coriandrum sativum* L.

Table 2. Metaphasic and Anaphasic Abnormalities induced by UV-B radiations in root meristems of *Coriandrum sativum* L.

TREATMENT (UV-B)	DOSES (Minutes)	METAPHASIC ABNORMALITY (%) (Mean ± S.E.)					ANAPHASIC ABNORMALITY (%) (Mean ± S.E.)				
		ST	CM	PR	SC	UN	BRDG	ST	UN	LG	OTH
1st DAY	20	0.63 ± 0.12^{a}	0.13 ± 0.12^{a}	0.12 ± 0.12^{a}	0.25 ± 0.12^{a}	0.25 ± 0.12^{a}	0.50 ± 0.12^{a}	0.12 ± 0.12^{a}	0.38 ± 0.21^{a}	0.00±0.00	0.12 ± 0.12^{a}
	40	0.51 ± 0.09^{a}	0.39 ± 0.01^{a}	0.14 ± 0.13^{a}	0.39 ± 0.19^{a}	0.13 ± 0.10^{a}	0.27 ± 0.13^{a}	0.27 ± 0.13^{a}	0.51 ± 0.99^{a}	0.37 ± 0.20^{a}	0.27 ± 0.13^{a}
	60	1.05 ± 0.25^{a}	0.21 ± 0.10^{a}	0.23 ± 0.11^{a}	0.45 ± 0.96^{a}	0.34 ± 0.19^{a}	0.69 ± 0.19^{a}	0.00±0.00	0.32 ± 0.18^{a}	O.36±0.22^{a}	0.45 ± 0.10^{a}
2nd DAY	20	1.13 ± 0.21^{ab}	0.63 ± 0.12^{a}	0.12 ± 0.11^{b}	0.50 ± 0.12^{ab}	0.50 ± 0.13^{a}	0.38 ± 0.02^{b}	0.00±0.00	0.25 ± 0.12^{ab}	0.00±0.00	0.00±0.00
	40	0.65 ± 0.14^{b}	0.36 ± 0.20^{a}	1.27 ± 0.09^{a}	0.13 ± 0.12^{b}	0.50 ± 0.11^{a}	0.13 ± 0.12^{b}	0.13 ± 0.12^{a}	0.64 ± 0.13^{a}	1.02 ± 0.12^{a}	0.00±0.00
	60	1.67 ± 0.31^{a}	0.76 ± 0.03^{a}	0.23 ± 0.21^{b}	0.63 ± 0.11^{a}	0.26 ± 0.12^{a}	0.18 ± 0.19^{a}	0.24 ± 0.12^{a}	0.12 ± 0.11^{a}	0.36 ± 0.20^{b}	0.26 ± 0.13^{a}
3rd DAY	20	0.63 ± 0.12^{b}	0.50 ± 0.25^{a}	0.37 ± 0.21^{a}	0.37 ± 0.21^{a}	0.25 ± 0.12^{a}	0.62 ± 0.12^{b}	0.62 ± 0.12^{a}	0.37 ± 0.21^{a}	0.62 ± 0.12^{a}	0.25 ± 0.12^{b}
	40	1.88 ± 0.35^{a}	0.41 ± 0.24^{a}	0.00 ± 0.00^{a}	0.54 ± 0.15^{a}	0.66 ± 0.25^{a}	1.62 ± 0.27^{a}	0.39 ± 0.22^{a}	0.40 ± 0.02^{a}	0.53 ± 0.13^{a}	0.26 ± 0.21^{b}
	60	1.94 ± 0.11^{a}	0.91 ± 0.11^{a}	0.34 ± 0.19^{a}	0.34 ± 0.01^{a}	0.46 ± 0.30^{a}	1.83 ± 0.11^{a}	0.45 ± 0.29^{a}	0.57±0.23a	0.57 ± 0.11^{a}	1.14 ± 0.10^{a}

*****Abbreviations: **ST**-Stickiness, **CM**-C-metaphase, **PR**-Precocious movement, **SC**- Scattering, **UN**-Unorientation, **BRDG**-Bridge, **LG**-Laggards, **OTH**-Other abnormalities. Means followed by lowercase letter is statistically significant at $p<0.05$.

3.1.2. Effect on Chromosomal Organization

From present study it was recorded that as the treatment duration increases the rate of chromosomal aberrations was also increased. Mitotic disturbances after UV-B treatments were dominantly confined at metaphase and anaphase as shown in Figure 1. The rate of chromosomal aberrations was documented in the form of Total Abnormality Percentage (TAB %) in Table 1. It was increased from 2.53 ± 0.13^{c} to 4.16 ± 0.32^{a} on 1st day, 3.54 ± 0.12^{c} to 6.34 ± 0.12^{a} on 2nd day and 4.64 ± 0.33^{c} to 8.58 ± 0.37^{a} on 3rd day, respectively. From the results, it was inferred that higher doses are more genotoxic and impose more aberrations in treated sets as compared to lower doses and control. The wide range of chromosomal aberrations observed were precocious movement of chromosomes (Figure 1.D), c-metaphase (Figure 1.E), clumping (Figure 1.F), scattering (Figure 1.G) at metaphase while forward movement (Figure 1.H), unorientation (Figure 1.I), laggard (Figure 1.J) and multiple bridge (Figure 1.K) at anaphase, respectively. The percentage of chromosomal aberrations increased as the time and duration of supplemental UV-B treatment was increased. Stickiness was found to be most dominant anomaly (1.94 ± 0.11^{a}) induced by UV-B at metaphase recorded in 3rd day treatment at 60 minutes while bridges were recorded as 1.83 ± 0.11^{a} on 3rd day treatment, precocious movement (1.27 ± 0.09^{a}) and laggards were next to induced at a higher frequency in 2nd day treatment at 40 minutes duration. UV-B induces lesser telophasic and other cellular abnormalities. The trend of increasing Total Abnormality Percentage (TAB %) along with UV-B treatment was shown in Figure 3.

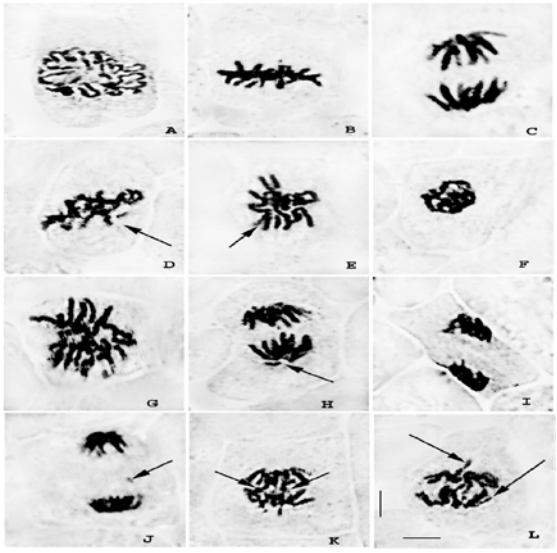

Figure 1. Different types of chromosomal aberrations induced by UV-B irradiation in root meristems of *Coriandrum sativum* L.- **Legends of figure- A:** Normal prophase**, B:** Normal metaphase **(2n=22), C:** Normal anaphase **(22:22), D:** Precocious movement of chromosomes with unorientation at metaphase**, E:** C-metaphase, **F**: Clumping at metaphase, **G:** Scattered chromosomes at metaphase, **H**: Forward movement at anaphase, **I**: Unorientation at metaphase, **J**: Laggard at anaphase, **K**: Multiple bridge at anaphase, **L**: Broken bridge with forward movement at anaphase.**[Scale bar: Length (1 cm) = 3.7 µm, Width (1 cm) =2.2 µm]**

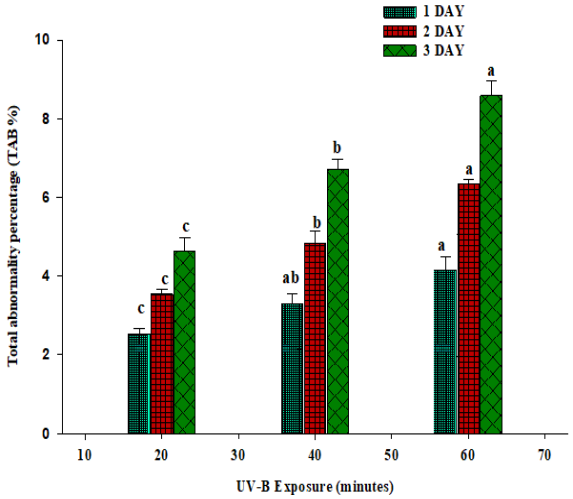

Figure 3.Comparative trend abnormalities induced at different doses of UV-B treatment on root meristems of *Coriandrum sativum* L.

3.2. Biochemical Observations

3.2.1. Effect on Proline Content

A sharp increase in levels of proline was observed in leaves of Coriander upon exposure to UV-B radiations. Along with the increase of time and duration of UV-B treatment the accumulation of proline content was also increased to 4.97 ± 0.70^{a} as compared to control 2.56 ± 0.36^{a}. Figure 4 shows the trend of proline content on 1st, 2nd and 3rd day UV-B treatment.

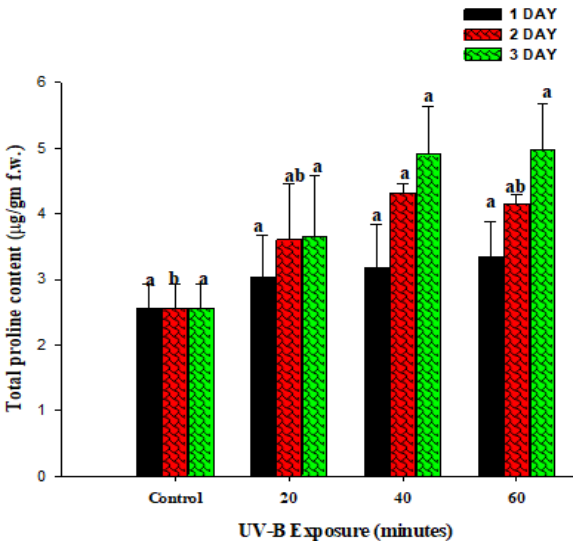

Figure 4. Showing an account of total proline content (mg/gm fw.) after UV-B treatment on 1st 2nd and 3rd day on *Coriandrum sativum* L.

3.2.2. Effect on Carbohydrate Content

There is a maximum level of increased carbohydrate content 6.51 ± 0.22^{a} at 60 minute in coriander over control 5.90 ± 0.34^{ab} on 1st day UV-B and it declined on 3rd day exposure. The trend of carbohydrate content in Coriander has been shown in Figure 5.

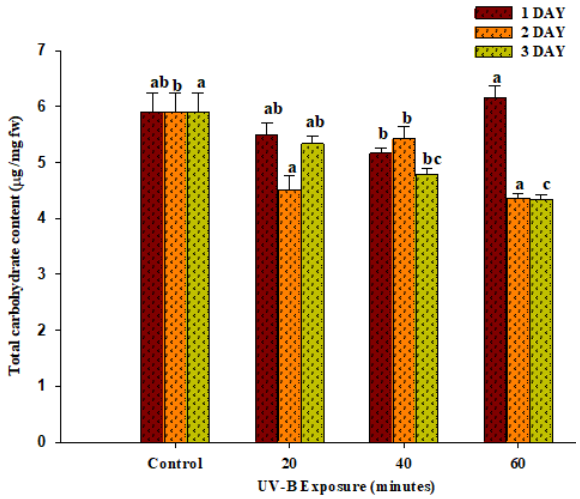

Figure 5. Showing the comparative trend of total carbohydrate content (mg/g f.w.) after UV-B exposure on 1st, 2nd and 3rd day treatment on *Coriandrum sativum* L.

3.2.3. Effect on Photosynthetic Pigments

The data scored envisaged that the supplemental UV-B radiation causes reduction in photosynthetic pigments of coriander leaves.

3.2.3.1. Chlorophyll a

Inhibition in chlorophyll a content was observed as the exposure time and duration of UV-B treatment was increased (Figure 6). Maximum inhibition (1.56 ± 0.27^{b}) was recorded at 60 minutes exposure on 2nd day treatment. A steady decline of 23% in chlorophyll a contents was observed on 1st day treatment over control.

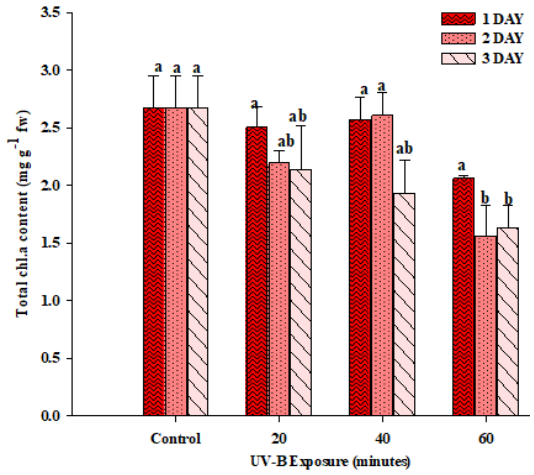

Figure 6. Showing the trend of chlorophyll a content (mg/g f.w.) after effect of UV-B treatment for 1st, 2nd and 3rd day exposure on *Coriandrum sativum* L.

3.2.3.2. Chlorophyll b

It was found to be increased by 5% over control set and reached to maximum level of 1.82 ± 0.10^{a} as compared to control 1.73 ± 0.24^{a} (Figure 7). An abrupt decline (1.07 ± 0.02^{b}) was recorded on 2nd day treatment at 40 minute exposure over control.

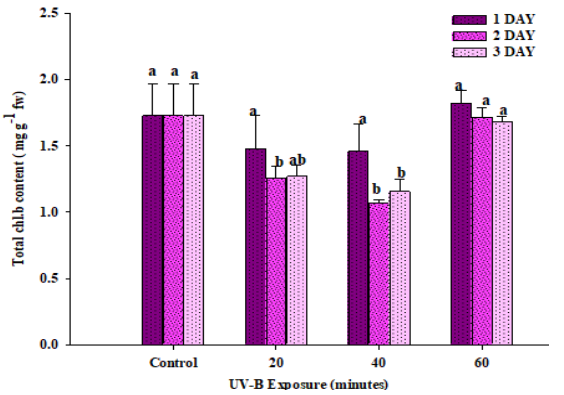

Figure 7. Showing the trend of chlorophyll b content (mg/g f.w.) of *Coriandrum sativum* L. after UV-B treatment for 1st, 2nd and 3rd day exposure duration of 20, 40 and 60 minutes.

3.2.3.3. Carotenoid

A significant decrease in carotenoid contents was recorded in UV-B treated sets over control Figure 8. A maximum increase was recorded in 20 minutes treated sets on 1st and 2nd day treated sets. However, there is constitutive decline by 27% on 1st day, 35% on 2nd day and 72% on 3rd day.

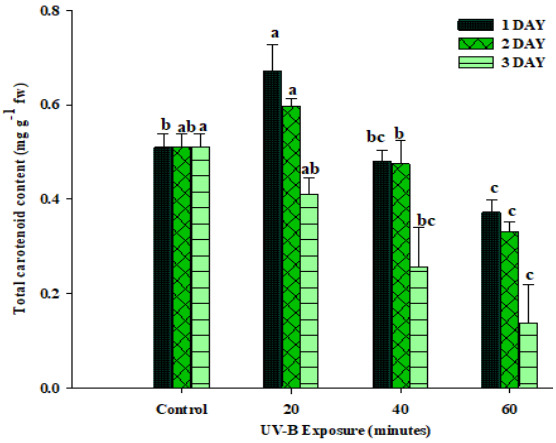

Figure 8. Showing an account of total carotenoid content (mg/g f.w.) after 1st, 2nd and 3rd day impact of UV-B exposure on *Coriandrum sativum* L.

4. Discussion

UV radiation plays an important regulatory role in plants growth and development. However, due to its putative functioning higher exposure of UV-B can cause stressful conditions leading to damaging impacts on physiological process and genome instability which in turn concerned with the human health and also plant production and quality measures.

In the present study, we have investigated the effect of UV-B on the root meristems of *Coriandrum sativum* L. with a wide range of doses and compared its action on 1st, 2nd and 3rd day exposure duration.

4.1. Cytological

The data gathered from present study show a significant decrease in mitotic index along with dose dependent increase in chromosomal aberrations in sets raised with

supplemental UV-B radiations. Similar results were recorded by Csilla (2009) and Hopkins *et al.* (2002).

Slowing of mitosis results in decreased MI % which is a protective mechanism acquired by plants to cope up with higher doses of UV-B radiation stress as DNA is most sensitive to UV-B during replication. According to Liu *et al*. (2015) reduced MI may be the outcome of breakdown of plant self-protection system and further inhibition of cell DNA replication, transcription and protein synthesis. In *Picea abeis*, Bavcon and Gogala (1996) also reported a decreased mitotic activity and lesser vitality due to influence of UV-B radiation. Arrested interphase due to damaging action of UV-B might leads to decline in cell division. Due to decreased ATP levels and pressure excursion by energy producing centre, probably inhibits the DNA synthesis and reduced ATP causing low MI (%). Reduced mitotic index is due to chromosome condensation in early prophase of the mitotic cycle, but prior to breakdown of nuclear membrane. It could be revealed that declined MI is due to mitodepressive actions of higher UV-B exposure duration and inhibition of DNA synthesis at telophase (Sudhakar *et al*., 2001). UV-B radiations are known to be a physical mutagens producing wide variety of chromosomal aberrations producing abnormal cells. In present investigations a vast spectrum of mitotic chromosomal aberrations were recorded in sets raised to UV-B treatment in Coriander.

CAs is of 2 types, chromosomal and chromatid type, but increased pool of chromosome type aberrations elucidates the genotoxic activity of UV-B radiation. Similar results were recorded by Ranceliene and Vysniauskiene, 2012. According to Cieminis *et al*. (1987), UV-B induced photoproducts could induce the formation of chromosomal aberrations; some of them could be cyclobutane-pyrimidine dimmers that prove to be a genetical danger for plants. Stickiness was found to be most dominant anomaly at metaphase chromosomal stickiness leads to inactivation of DNA replication, increased chromosomal contraction and condensation or nucleoproteins probably leading to cell death (Han *et al*., 2007). It could be due to depolymerisation of nucleic acid caused by mutagenic treatments or due to partial dissociation of the nucleoproteins and alterations in their pattern of organisation (Evans, 1962). Precocious movement of chromosomes at metaphase might be formed due to malformed homology of chromosome pairing or spindle mechanism whereby one or few chromosomes floats in the cytoplasm rather than arranged at equatorial plate. Probably, the disrupted spindle functioning causes precocious chromosomes. Spindle disruption also causes scattering, unorientation and c-metaphase. C-metaphase was first reported by Levan, 1938 in root tips of *Allium cepa* caused by inactivation of the spindle fibre followed by a random scattering of chromosomes over the cell. Unorientation and scattering of chromosomes at metaphase was observed in the present investigation which may be either due to inhibition of spindle fibre formation or destruction (Kumar and Rai, 2007). Chromatin bridges were another anomaly encountered dominantly at anaphase. It may occurred due to enhanced activity of UV-B radiations, making chromosome breaks, then the two chromosome sides are, respectively, healed, producing double centromere chromosomes i.e. "chromosome bridges." Bridges was also reported by Dhulgande (2015). Formation of bridges could be attributed to chromosomal stickiness (El-Khodary *et al*., 1990) and to chromosome breakage and reunion (Haliem, 1990) that may lead due to loss of genetic material. The loop forming laggards at anaphase (Figure 1.H) might have originated due to failure of kinetochores to attach with spindles and leading to the joining of ends forming loops. Such disorders may lead to mutations. A merotelic kinetochores orientation is a major cause of lagging chromosomes during mitosis. It was suspected that those chromosomes which do not active in bridge formation may sometimes get detached from the group and are remained as lagging in cell vicinity. DNA damages induced by UV-B radiation might have influenced the expression of number of genes leading to alterations in proteins that control many metabolic processes like plant Development, cell cycle, fertilisation and seed formation (Haliem *et al.*, 2013).

4.2. Biochemical Observations

4.2.1. Proline Content

Data of present study elucidate that the different time and duration of UV-B radiation induces increased proline contents in all treated sets as compared to control sets in *Coriandrum sativum* L. The findings of Demir (2000) and Amal *et al*. (2006) are in agreement with the present findings. In the seedlings of rice and mungbean accumulation of proline due to UV-B radiation has been reported. Masood *et al*. (2006) had also reported increased proline content in *Azolla pinnata* and *A. filiculoides* under UV-B treatment. Liang *et al*. (2013) and Saradhi *et al*. (1995) stated that accumulated proline is an adaptive measure of plants against adverse conditions and it involves stabilization of proteins and antioxidant enzymes, direct scavenging of ROS, balance of intracellular redox homeostasis (ratio of $NADP^+$/NADPH and GSH/GSSG) and cellular signalling promoted by proline metabolism and suppression of mitochondrial electron transport might be cause of proline accumulation. Stimulation of proline from abscissic acid, inhibition of proline oxidation to other soluble compounds and inhibition of protein synthesis are the causes of free proline accumulation.

4.2.2. Carbohydrate Content

These are the key source of energy for plants basic life functions. They harvest it by capturing incident solar radiation *via* photosynthesis. But elevated UV-B radiation disrupts the machinery significantly results in decreased soluble carbohydrates. The findings of Moghadam *et al*.(2012) are in agreement with the present findings. It may be suspected that UV-B distorts the grana causing inhibited photosynthesis leading to decreased rate of carbohydrate formation as exposure level increases. As UV-B treated plants have a tendency to lower sink capacity (Correia *et al.*, 2000), the observed decreased in the total carbohydrate content by UV-B indicates the main response is mediated by lower rate net photosynthetic rate (Correia *et al*., 2005). Similar results were observed by Musil (1996) and Mackerness *et al.* (1997). At 60 minutes, carbohydrate content gets increased on 1st day (Table: 3) but latter declined. Kovacs *et al*., 2002 stated that supplementary UV-B radiation damaged the structure of chloroplasts, as manifestated by dilations of thylakoids, a

progressive disruption of thylakoid structure and disintegration of the double membrane envelope surrounding the chloroplast, accompanied by the accumulation of large starch grains at higher level due to immobilization and then later declined.

4.2.3. Photosynthetic Pigments

Pigments of photosynthetic apparatus can be destroyed by UV-B radiation with comparative loss of photosynthetic capacity (Jordan *et al*., 1994). Chlorophylls and carotenoids were affected by differential UV-B radiation doses, while carotenoids are generally less affected than chlorophylls (Pfundel *et al.*, 1992). It has been reported that in tested plant (Table 3) the Chlorophyll a decreased as exposure duration increases but on 40 minutes treatment sets UV effect was regressed by plant but at 60 minutes causes reduction in chlorophyll a contents as compared to Chlorophyll b (Table 3). Similar findings were reported by Marwood and Greenberg (1996) that might point as more selective destruction of Chlorophyll a biosynthesis or degradation of precursors probably decrease in PSII due to higher UV-B radiations caused decreased Chlorophyll a. Decreased carotenoids may play a role in the decrease of chlorophyll concentrations since carotenoids protect chlorophyll from photo-oxidative damages (Singh, 1996). As compared to control at lower dose carotenoids increased to protects plants against UV-B radiation but at higher doses level decreases. The reduction in carotenoid content may result either from inhibition of synthesis or from breakdown of the pigments. Since carotenoids are involved in the light harvesting and protection of chlorophylls from photo-oxidative damages, any reduction in carotenoids could have serious consequences of chlorophyll pigments (Ravindran *et al*., 2010).

Table 3. Effect of UV-B radiations on proline, carbohydrate and photosynthetic pigments (chl a, chl b, & carotenoid) of *Coriandrum sativum* L.

Treatment (UV-B)	Doses (minutes)	Proline (µg/gm f.w.)	Carbohydrate (µg/gm f.w.)	Chl a* (mg/gm f.w.)	Chl b** (mg/gm f.w.)	Carotenoid (mg/gm f.w.)
1 DAY	Control	2.56±0.36^{a}	5.90±0.34^{ab}	2.67±0.28^{a}	1.73±0.24^{a}	0.51±0.03^{b}
	20	3.04±0.63^{a}	5.50±0.20^{ab}	2.51±0.17^{a}	1.48±0.25^{a}	0.67±0.05^{a}
	40	3.17±0.66^{a}	5.17±0.08^{b}	2.57±0.19^{a}	1.46±0.21^{a}	0.48±0.02^{bc}
	60	3.33±0.55^{a}	6.15±0.22^{a}	2.05±0.02^{a}	1.82±0.10^{a}	0.37±0.03^{c}
2 DAY	Control	2.56±0.36^{b}	5.90±0.34^{a}	2.67±0.28^{a}	1.73±0.24^{a}	0.51±0.03^{ab}
	20	3.60±0.86^{ab}	4.51±0.26^{b}	2.19±0.11^{ab}	1.26±0.09^{b}	0.59±0.02^{a}
	40	4.32±0.14^{a}	5.42±0.21^{a}	2.61±0.20^{a}	1.07±0.02^{b}	0.47±0.05^{b}
	60	4.16±0.13^{ab}	4.36±0.08^{b}	1.56±0.27^{b}	1.72±0.07^{a}	0.33±0.02^{c}
3 DAY	Control	2.56±0.36^{a}	5.90±0.34^{a}	2.67±0.28^{a}	1.73±0.24^{a}	0.51±0.03^{a}
	20	3.65±0.93^{a}	5.34±0.15^{ab}	2.14±0.37^{ab}	1.27±0.08^{ab}	0.41±0.03^{ab}
	40	4.91±0.73^{a}	4.78±0.10^{bc}	1.93±0.28^{ab}	1.16±0.09^{b}	0.26±0.08^{bc}
	60	4.97±0.70^{a}	4.34±0.09^{c}	1.62±0.19^{b}	1.68±0.04^{a}	0.14±0.08^{c}

1-Abbreviations*- Chlorophyll a, **- Chlorophyll b

2- Data are represented in Mean ± S.E. significantly different at $p<0.05$.

5. Conclusion

The results obtained from the present experimental work elucidate that higher doses of UV-B induces various cytological anomalies resulting into decreased MI (%) and exaggerated chromosomal aberrations as exposure duration increases. However, as concerned to biochemical responses of *Coriandrum sativum* L. against UV-B radiation the level of proline was increased to protect plant machinery but chlorophyll pigments (chl a, chl b and carotenoids) and total carbohydrate contents were declined which is correlated with decreased photosynthetic rates due to aberrations in cellular division rates in grana causing disruption and altered signalling during mechanism of photosynthetic process.

Hence, it can be concluded from the present result that the lower doses and exposure duration of UV-B are less significant in its action and promote some important metabolic processes in plants while higher doses are significant and induces toxicity. The present research work would be further helpful in selecting the doses which imparts characters of interest in *Coriandrum sativum* L. and for further study on UV-B responses of *Coriandrum sativum* L. after raising generations up to complete maturity stage and will provide an insight to understand the impact of UV-B on commercial crops and to devise necessary protective measures and strategy for conservation of spice crops of interest like coriander and provide awareness among farmers for ill effects of UV-B on crops.

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Flight Activity of the Hairy Rose Beetle, *Tropinota squalida* (Scopoli) in Apple and Cherry Orchards in Southern Jordan

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Abstract

The hairy rose beetle, *Tropinota squalida* (Scopoli), is one of the important insect pests that attack pome and stone fruits in the southern part of Jordan. This destructive pest attacks the flowers of the crop where it feeds on the reproductive parts of the flowers. The present study was initiated to monitor the phenology and flight activity of the hairy rose beetle in an attempt to provide basic information for the development of safe and effective control measures for this economically important insect pest. The present study was conducted during 2009-2010 on two apple cultivars, Grany Smith and Royal Gala, and on Stella cherry. Capture of the beetles was performed via locally constructed traps that were improved by incorporation of the floral chemical attractants. Adults of the beetle were captured in apple orchards earlier than cherry orchards and disappeared earlier in the cherry orchard than that in the apple orchards. The flight activity of beetle started in the last week of January to the end of May in the apple orchards, but it was during mid-February to mid of May in cherry orchard. Positive relationships were obtained between the beetle flight activity the flowering pattern of the studied fruit crops.

Key words: *Tropinotasqualida*, hairy rose beetle, flowering, flight activity.

1. Introduction

The southern part of Jordan, mainly Ash-Shoubak region, 220 km south of Amman, is considered the main apple growing area in Jordan. The total area planted with apples is 4905 ha., with an annual production of 22833 ton (Ministry of Agriculture, 2014). Beside apples, other fruit trees, such as plum, cherry, pears and apricots, are also abundant in the region. Many insect pests were recorded to attack apples in Ash-Shoubak, such as the codling moth, *Cydia pomonella* (Madanat and Al-Antary, 2012), woolly apple aphid, *Eriosoma lanigerum* (Ateyyat *et al*., 2011; Ateyyat, 2012), the small red belted clear wing borer, *Synanthedon myopaeformis* (Ateyyat, 2006; Ateyyat and Al-Antary, 2006). One of the important insect pests of apples and other fruit trees in southern Jordan is the hairy rose beetle, *Tropinota squalida* (Scopoli). This destructive pest attacks the flowers of the crop where it feeds on the reproductive parts of the flowers. The feeding usually results in the damage of the anthers and stigma which affects fruit setting and prominently reduces the yield (Abdel-Razek and Abd-Elgawad, 2008). As for many insects that attack the crop during flowering, the hairy rose beetle is difficult to control (Vuts *et al.*, 2010). Currently, growers rely on two control tactics: hand picking of the adult beetle from the infested trees and application of chemical insecticides. Hand picking is practiced when few numbers are found on the flowering crop while insecticidal applications are implemented when large numbers infest the trees. Hand picking is time and labor consuming which makes it impractical when large numbers attack the trees. On the other hand, insecticidal applications should be avoided during flowering to prevent flower drop from spraying pressure and to conserve pollinators that are abundant on the trees during flowering (Schmera e*t al.*, 2004). Moreover, chemical insecticides are well known for their hazards to humans and the environment.

Many beetles in the subfamily Cetoniinae including the hairy rose beetle use both visual and olfactory stimuli from the flowers of the crop to locate its host. Depending on these stimuli, a funnel trap was developed to capture a closely related species; *Epicometis (Tropinota) hirta* and found effective in capturing the beetle (Ali, 1993; Schmera *et al.*, 2004). Afterwards, the trap was shown to effectively capture the hairy rose beetle (Toth *et al.*, 2009). The visual stimuli are affected by the color of the trap where the blue color was found the most attractive (Schmera *et al.*, 2004).

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Capture of the beetle with blue traps was considerably improved by incorporation of the floral chemical attractants: cinnamyl alcohol and trans-anethole at a ratio of 1:1 (Schmera *et al.*, 2004).

There are enormous studies on the use of trapping as a mean for monitoring insect flight activity. Similarly, the hairy rose beetle can be detected and monitored using the funnel trap baited with the floral attractants (Schmera *et al.*, 2004). Determination of the phenology and flight activity is a corner stone in the management of the hairy rose beetle. It will avoid unnecessary insecticidal applications and provide information on the magnitude of pest attack as well as the proper timing for the implementation of control measures. Therefore, the present study was initiated to monitor the phenology and flight activity of the hairy rose beetle in an attempt to provide basic information for the development of safe and effective control measure for this economically important insect pest.

2. Materials and Methods

The experiments were conducted in Al-Hashlamoun apple orchards (about 120,000 apple trees) during January 2009 to the end of May 2010 in Ash-Shoubak area (about 1300 m above sea level and 220 km south of Amman, the capital of Jordan). Orchardists were requested not to interfere with any pesticide application.

The trees at the experiment site were planted in 1994, and trained under the central-leader system to an average height of 3 m (range 2.5.–3.5 m), with a mean butt diameter of approximately 30 cm. All apple trees were planted on Merton-Malling Series (MM) 106 rootstock. Planting distances were based on a 5 by 5 m grid. The experiment was conducted on two cultivars of apple of different characteristics; Royal Gala and Granny Smith. Also,the flight activity of hairy rose beetle was studied in Stella cherry orchard in which trees were planted in 1996.

The hairy rose beetle trap (HRBT, Fig. 1)) was used to catch the adults of the insect pest. This trap consists of three main parts: a landing and collecting platform, a container, and an attracting dispenser. The landing platform is a funnel (21 cm diameter X 25 cm height) made from plastic. The container serves to hold the captured insects that fill into the trap and was made from empty pesticides plastic bottles (1L. in size). The crew cap of the bottles constituted the base of the container so that it can be opened and emptied if the trap is full of beetles. The landing platform and the container were adhered together using plastic glue. The dispenser was prepared using the floral attractants of another species, *Epicometis* (*Tropinota*) *hirta*. The attractants were (E)-cinnamyl alcohol (3-phenyl-2-propenyl alcohol) and (E)-anethole ((E)-4-propenylmethoxybenzene) in 50% dichloromethane solution (Sigma Aldrich, Eu.) at a ratio of 1:1 (Toth *et al.*, 2004). One hundred mg of each attractant were added to 2 x 2 x 1 cm cotton pieces. The cotton pieces were then placed in 150µm polyethylene bags and the bags were heat-sealed. The dispensers were individually wrapped in aluminum foil and stored at -20ºC until used. When used in the field, the dispensers were adhered to the inner surface of the funnel using grey duct tape and then punctured 5 times using size 2 insect pin.

Figure 1. Hairy Rose Beetle Trap

For each plant species/cultivar, 3 traps were placed between rows of trees. The traps were white in color and supplied with one dispenser. They were separated by approximately 100 m. The traps were fastened on 100cm wooden sticks so that the base of the trap was approximately 20 cm above ground. Numbers of captured adults of the hairy rose beetle were counted in each trap weekly post placement of the traps. SAS program (2012), version 9 was used to find Pearson correlation data of weather factors (minimum, maximum and average temperatures, and relative humidity) with number of captured adults of *Tropinota squalida* on Grany Smith and Royal Gala apples and Stella cherry orchards in Ash-Shoubak area.

3. Results and Discussion

The effectiveness of the employed sampling method has a strong effect on the quantification of an ecological community (Campos *et al.*, 2000). The trap effectiveness in catching Hairy rose beetle was studied previously by Al-alawi *et al.* (unpublished work) in the same experimental site. These field trials showed that the beetles were highly attracted to blue, white and yellow traps compared to green and orange colors. White traps placed on the ground attracted more beetles than traps hanged on the tree. Accordingly, we used white traps that were placed on the ground as described above.

In 2009, the flight activity of the Hairy rose beetle started within the first one-third part of February on Granny Smith orchard, and after one week traps installed in the Royal Gala orchard started to capture the adults of the beetle (Fig. 2). Flight activity of beetles delayed in

Stella cherry orchard in which traps started to capture adults within the first week of March (Fig. 2). No adults were captured after May 3 in the cherry orchard, but traps continued to capture adults until May 11 in the two apple orchards. The flight activity of the Hairy rose beetle was shown to be shorter in the cherry orchard (10 weeks) than that in the two apple orchards, Granny smith and Royal Gala (14 and 13 weeks, respectively). This might be attributed to variations in flowering periods that was shorter in cherry compared with apple. The highest captured numbers of adults were on April 13 in the Granny Smith and Royal Gala apples, and Stella cherry in which 287, 224 and 368 adults per trap were caught, respectively.

In 2010, adults were captured in apple orchards earlier than that recorded in 2009, in which they started their flight activity within the last week of January (Fig.3). The same scenario was obtained with Stella cherry orchard as adult flight activity was recorded after mid-February (Fig. 3). One distinguished peak was recorded for both apple cultivars that was on March 8 in which 459.3 and 478.3 adult/trap were caught in Granny Smith and Royal Gala orchards, respectively. The highest peak of flight activity was recorded within the last week of March in the cherry orchard (497.5 adults/trap). Flight activity of beetles continued until May 26 in the apple cultivars, but it stopped earlier in the Stella cherry orchard in which the last recorded activity was in mid-May (Fig. 3). In Egypt, the flight activity of *T. squalida* was reported during the last week of January to the last week of April of 2005/2006 (El-Sayed-Darwish, 2007).

It was noticed that the appearance and disappearance of *T. squalida* adults coincided with the appearance and disappearance of flowers of plants in the studied fruit crops. Attempts to find the correlations between the flight activity of the hairy rose beetle and both the temperature and relative humidity, showed very weak correlations (Table 1; Figs. 2 and 3). These results disagree with Hassanein and Salman (2009) who found that the minimum temperature and relative humidity are the main weather factors affecting the density of *T. squalida*. However, it was noticed that winds played a major effect as low numbers of adults were captured during the weeks in which windy days were recorded.

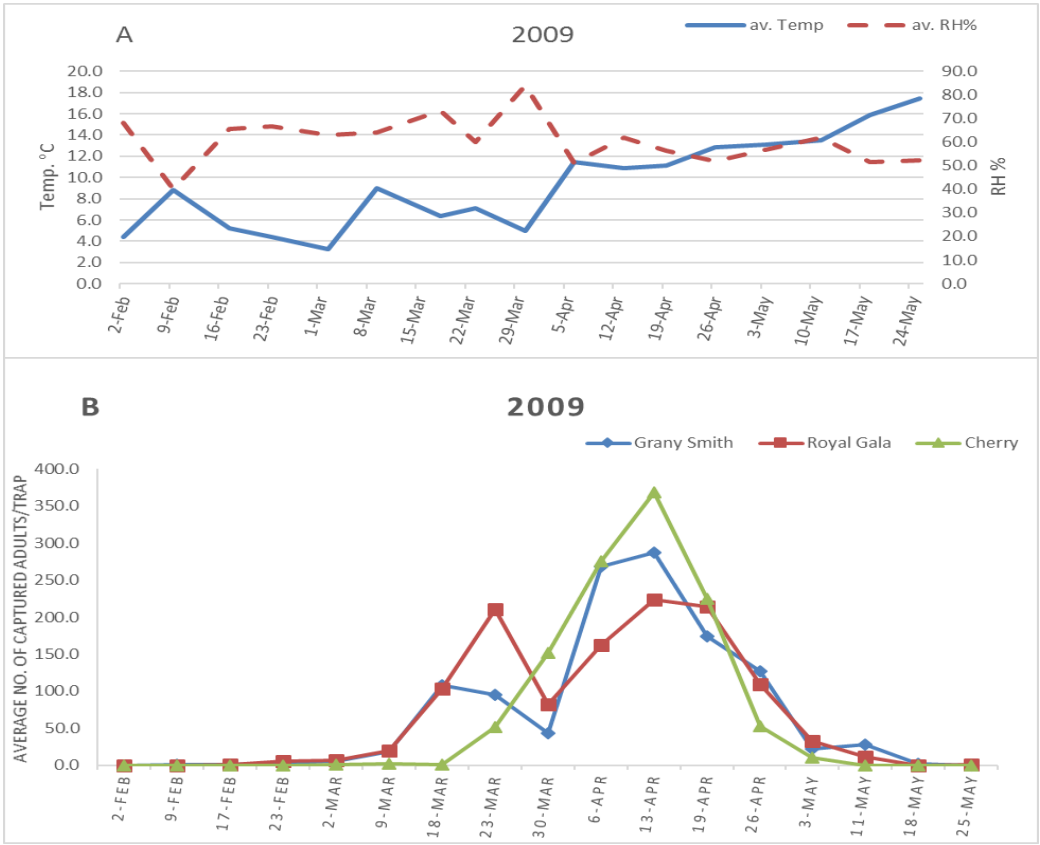

Figure 2. (A) Average temperature (°C) and average relative humidity (RH%) in Ash-Shoubak area during 2009. (B) Number of captured adults of *Tropinota squalida* on Grany Smith and Royal Gala apples and Stella cherry orchards in Ash-Shoubak area in 2009.

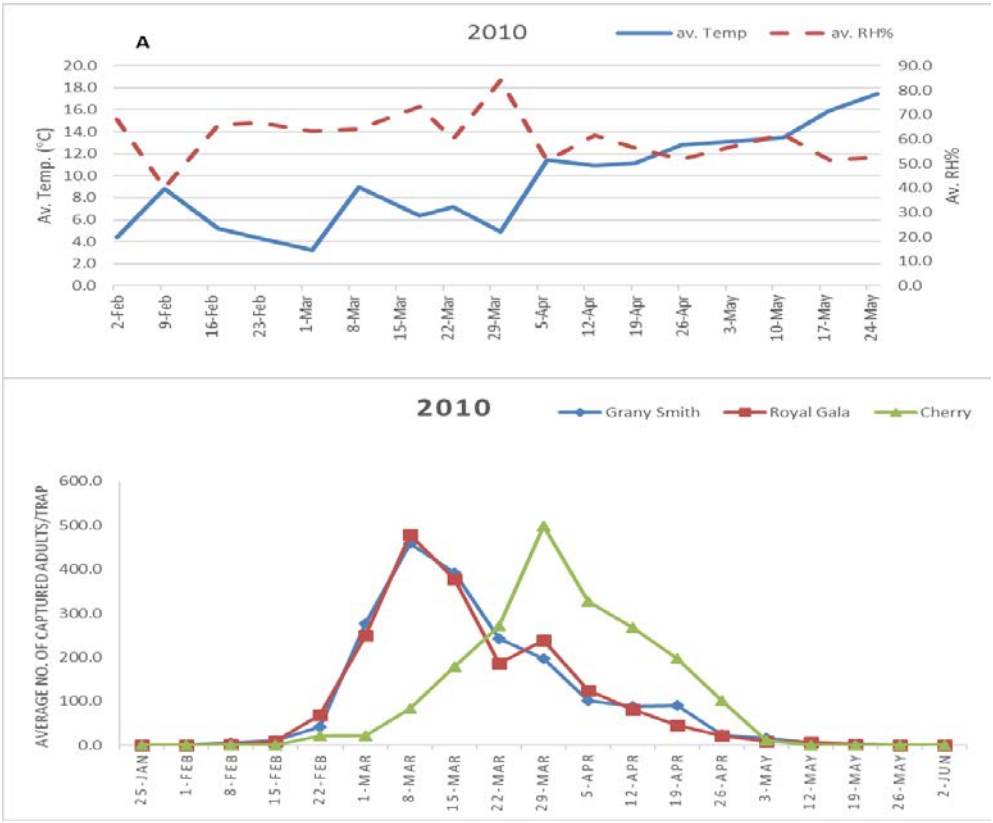

Figure 3. (A) Average temperature (°C) and average relative humidity (RH%) in Ash-Shoubak area during 2010.(B) Number of captured adults of *Tropinota squalida* on Grany Smith and Royal Gala apples and Stella cherry orchards in Ash-Shoubak area in 2010.

Table 1: Pearson correlation data of weather factors (minimum, maximum and average temperatures, and relative humidity) with number of captured adults of *Tropinota squalida* on Grany Smith and Royal Gala apples and Stella cherry orchards in Ash-Shoubak area

Pearson Correlation Coefficients, N = 35 Prob> |r| under H0: Rho=0						
	min temp	max temp	av. Temp	min RH%	max RH%	av. RH%
Grany Smith	0.11856	0.00913	0.05556	0.30977	-0.26353	0.09153
	0.4976	0.9585	0.7512	0.0702	0.1261	0.6010
Royal Gala	0.08717	-0.02353	0.02194	0.39594	-0.32718	0.12305
	0.6185	0.8933	0.9004	0.0186	0.0550	0.4813
Stella Cherry	0.10322	0.02098	0.05517	0.15359	-0.04663	0.09462
	0.5552	0.9048	0.7529	0.3784	0.7902	0.5887

In Jordan, *T. squalida* is a monovoltine pest that overwinters in soil as adults and emerges in early to mid Feb. to feed on male and female organs of the flowers of some plants such as fruit trees. Adults are known also to occasionally feed on young shoots, leaves and even fruits. Because of their high flying capabilities, they land on many different types of plants and continue feeding. As a result, damaged flowers cannot produce fruits (Anonymous, 2008; Özbek, 2008). Özbek *et al.* (1998) reported that coping with these insects is very challenging because blossoms are damaged; however, pesticides may be employed if the population becomes excessive. However, the use of pesticides using pressure sprayers during bloom usually results in flower dropping before pollination. In addition, insecticides have negative impacts on the environment and their application during the flowering period of the crop is limited due to the presence of pollinators foraging the crop during bloom (Schmera *et al.*, 2004). Al-Alawi (2014) showed that using the essential oils of both eucalyptol and fir plants caused valuable mortality to the hairy rose beetle with low toxicity to honey bees, but it is difficult to use these oils at large scale plantations. Therefore, mass trapping of adults using the HRBT that showed valuable catching ability to adults provides safe and pollinator friendly control measure for effective management of the target pest. In Jordan, these traps could be installed in the fields in mid-January to the end of May. For effective control of this insect pest, the following studies are required: (1) Determine the

recommended density of the traps per unit area; (2) studying the correlation to wing speed with the flight activity; (3) Searching for the hosts or microhabitats of larvae in order to destroy the pest during this stage if that is possible; and (4) doing some ecological studies related to the migration distances of adults.

4. Conclusions

The findings obtained from the present study about the phenology and flight activity of the hairy rose beetle provide valuable information that might form the basis for establishing benign control measures such as mass trapping and better timing of application of these tactics to maximize its effects. It also opens new avenues for more research on the different biological and ecological aspects of this economically important insect pest, taking into consideration the scarcity of the published studies on this insect, particularly on fruit trees.

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Putative Mechanism of Cadmium Bioremediation Employed by Resistant Bacteria

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Abstract

Cadmium is one of the non-essential and toxic heavy metals which affect the terrestrial and aquatic biota along with human beings due to its release from industrial effluents directly into terrestrial and aquatic ecosystem. The bioremediation of heavy metals using microorganisms has emerged as a substitute for the physicochemical techniques in recent years. So, the present study deals with the isolation and screening of heavy metal resistant bacteria from three different locations of battery manufacturing sites of Faridabad industrial area, Haryana, India. In this study, five bacterial isolates were selected based on high level of heavy metal resistance. Screening of the bacterial isolates for metal resistance against Cd^{2+}, Ni^{2+}, Hg^{2+}, Cu^{+2} and Pb^{2+} was done by determining the minimal inhibitory concentration ranging from 10µg/ml to 250µg/ml. All the isolates were screened for their plasmid profile. The size of the isolated plasmid DNA was found to be more than 10,000bp. To determine whether the resistance gene was solely encoded by the plasmid, plasmid curing was done using ethidium bromide. The results showed that the bacterial growth on Cd-supplemented medium was not completely inhibited after plasmid curing, indicating the presence of multimechanisms involved in conferring resistance. It was observed that extracellular polymeric substances produced by isolates MF1 and MF2 play an important role in metal sorption and constitutes a passive method in which the metal cations bind to the negative charges of acidic groups from exopolysaccharide. In the remaining isolates, cadmium is precipitated as cadmium sulfide through hydrogen sulfide production. These heavy metal resistant organisms hold promise for bioremediation of heavy metal polluted environment

Keywords: Bioremediation, heavy metals, metal resistant bacteria, hydrogen sulfide, plasmid

1. Introduction

The growing industrialization has spread worldwide and has left persistent toxic heavy metals, like chromium, nickel, lead, zinc, cadmium and copper in our ecosystem. These heavy metals tend to accumulate and deteriorate the environment. This is especially true for developing countries like China and India (Raja *et al*., 2008). Common sources of heavy metal pollution include discharge from sources such as electroplating, plastic manufacturing industries, fertilizer producing plants and wastes left after mining and metallurgical processes (Zoubonlis *et al*., 2004). The heaviest metals exist naturally in the earth's crust at trace concentrations of just a few parts per million (Bodek *et al*., 1988), sufficient to provide local biota with trace nutrients, but too low to cause toxicity. Disposal of wastes from metal excavation and processing has increased the concentration of these heavy metals to dangerous levels in some soils. Cadmium is a heavy metal recognized as one of the most hazardous environmental pollutants (Lodeiro *et al.*, 2005), which is toxic to humans and aquatic life. Chronic exposure to cadmium can affect the nervous system, liver, cardiovascular system and may lead to renal failure and even death in mammals and humans (Semerjian, 2010). The use of conventional technologies, such as ion exchange, chemical precipitation, reverse osmosis and evaporation recovery for this purpose is often inefficient and or very expensive (Pratik and Hitesh, 2014).The bioremediation of heavy metals using microorganisms is not only a scientific novelty but it is also known for its potential application in industry (Singh *et al.*, 2010). In order to survive in heavy metal polluted environments, many microorganisms have developed a resistance to toxic metal ions (Kumar *et al.*, 2011). Several resistance mechanisms, such as metal efflux, intracellular sequestration by exopolysaccharide cell surface, biosorption by negative groups, bioprecipitation and redox reaction, have been found to be present in microorganisms to counteract heavy metal

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stress (Naik *et al.*, 2012). However, most mechanisms reported involve the efflux of metal ions outside the cell and the genes for tolerance mechanism have been found on both chromosomes and plasmids. Many bacterial strains contain genetic determinants of resistance to heavy metals such as Hg^{2+}, Ag^{2+}, Cu^{2+}, Ni^{2+}, Cd^{2+} and others (Karelova *et al*., 2011). These resistance determinants are often found on plasmids and transposons (Silver and Misra, 1988). The present study was undertaken with the aim of isolating the cadmium resistant bacterial isolates and to study their probable mechanism conferring the resistance. Such microbial populations specifically adapted to high concentrations of heavy metals hold promise for bioremediation of heavy metals from industrial effluents and soil.

2. Material and Methods

2.1. Sample Collection

Soil samples were taken from different locations of battery manufacturing contaminated environment in Faridabad industrial area of Haryana state in India. Soil samples (three to four) were collected randomly from the single site and pooled together. All the samples collected this way from the other sites were stored in sterilized polypropylene bags and kept in the refrigerator at 4^0C.

2.2. Isolation of Cadmium Resistant Bacteria

For isolation of cadmium resistant bacteria, dilution plating was performed; 10-10,000 fold dilutions of fresh soil (1g) were made in sterile distilled water and 0.1 ml from each of these dilutions was placed on Luria Bertani agar (10g peptone, 5g yeast extract, 10g NaCl, 15g agar, pH 7.2) (HiMedia Laboratories Pvt. Ltd.) plates containing 10µg/ml cadmium as cadmium nitrate. The plates were then incubated for 24-48h at 30^0C. Individual colonies of bacteria which varied in shape and color were picked up and further purified. Purified isolates were biochemically characterized as per Bergey's Manual of Systemic Bacteriology.

2.3. Growth Curves of Bacterial Isolates with Metal Induction

An aliquot of 100µl of each bacterial culture was inoculated separately in 100ml of Luria Bertani broth (with and without cadmium nitrate-20µg/ml), and these were incubated at 30^0C in rotary shaker at 500rpm. Aliquots of the culture were taken out in sterilized tubes, at regular intervals of time (6, 12, 24, 48and 72h). Growth was monitored by measuring the Optical Density (OD) at 620nm.

2.4. Determination of Minimum Inhibitory Concentration (MIC)

Minimum inhibitory concentration of the metals was determined by plate dilution method as described by Malik and Jaiswal (2000). The metals Cd^{+2}, Pb^{+2}, Hg^{+2} ,$Cu^{+2}$and Ni^{+2} were used as $Cd(NO_3)_2$, $Pb(NO_3)_2$, $HgCl_2$,$CuSO_4$ and $NiSO_4$, respectively. Stocks of the metal salts were prepared in distilled water and sterilized by filter membrane and stored at 4^0C. Luria Bertani medium was prepared and amended with various amounts of Cd, Pb, Hg, Cu and Ni to achieve the desired concentrations of 20, 40, 50, 60, 70, 80, 90, 100, 120, 140, 150,160, 180, 200 and 250µg/ml. Inoculums of all isolates were spread in the metal amended and control plates (without metal). The plates were incubated at 30^0C for 72h. The concentration of the metal, which permitted growth and beyond which there was no growth, was considered as the MIC of the metal against the strain tested.

2.5. Isolation of Plasmid and Plasmid Curing by Ethidium Bromide

The bacterial isolates were screened for the presence of plasmid DNA using the alkaline lysis method (Sambrook and Fritsch, 2001). The isolated plasmid was characterized by agarose gel electrophoresis at 70V for 2h. The gel was stained with ethidium-bromide, visualized under UV transillumination and photographed. For the plasmid curing, twenty four hour old cultures of the isolates were grown in sterile nutrient broth containing ethidium bromide (100µg/ml). The tubes were incubated at 30^0C for 24h. After incubation, the isolates were reinoculated in sterile nutrient broth and incubated further for 24h. The cured isolates were checked for their heavy metal resistance on LB agar plates having different concentrations of the various heavy metals.

2.6. Sulfide Production and Determination of Cadmium Sulfide Precipitation

For the determination of cadmium sulfide production, two experiments were performed. For a simple sulfide detection assay, a semisolid agar medium (Glucose-5.0g, Ammonium phosphate-1.0g, Sodium chloride-5.0g, $MgSO_4.7H_20$, 0.2% Noble agar) containing 2.5mM $FeCl_2.4H_2O$ and 3mM $Na_2S_2O_3.5H_2O$ (HiMedia Laboratories Pvt. Ltd.) was used and observed for the formation of a black precipitate (FeS) in the medium. In another experiment, selected isolates were grown in minimal broth supplemented with 3mm sodium thiosulfate and 50mM $CdCl_2$, incubated at 30^0C without agitation for the observation of orange precipitate.

2.7. Cadmium Accumulation and Removal Assay in the Presence of Thiosulphate

All the isolates were grown in 100 ml LB broth supplemented with 100µg/ml cadmium sulphate and LB broth supplemented with 3mM thiosulfate and 100µg/ml cadmium sulphate. The flasks were incubated on a shaker for 120h at 30^0C. Cells were harvested (One ml) at 24, 36, 48, 72 and 96h of incubation by centrifugation at 1100xg for10minutes at 4^0C. Bacterial cell residue was dissolved in 1ml 95% nitric acid mixed well by vortexing and diluted to 10ml with sterile double distilled water. Blanks were treated in the same way and analyzed by atomic absorption spectrometry. Cadmium was measured from the supernatant by the atomic absorption spectrometry. Percentage of Cd removal by the bacterial cells from the both culture (with and without thiosulfate) was calculated by taking difference between the initial metal content in the culture media and at the time of sampling. Cadmium removal studies were also done under the condition of glucose limitation. The selected isolates were grown in minimal media with and without sodium thiosulphate.

2.8. TEM Analysis

Bacterial cells were grown in media supplemented with cadmium (10µg/ml) for 48h and harvested by centrifugation and

observed in TEM at varying magnifications after treating by the standard methods.

3. Results

Five different bacterial isolates were isolated from the soil samples on LB media supplemented with 10µg/ml of cadmium as Cd $(NO_3)_2$. The morphological and biochemical characteristics of the isolates were studied and results were listed in Table 1. Results showed that two bacterial isolates (MF1 and MF2) were gram positive and three isolates (MF3, MF4 and MF5) were gram negative. Among all, isolates MF1 and MF2 were found to be negative for sulfide production. Growth curves for each of these isolates were studied in the presence of cadmium. Results showed that growth was not considerably affected in the presence of cadmium (Figure 1).

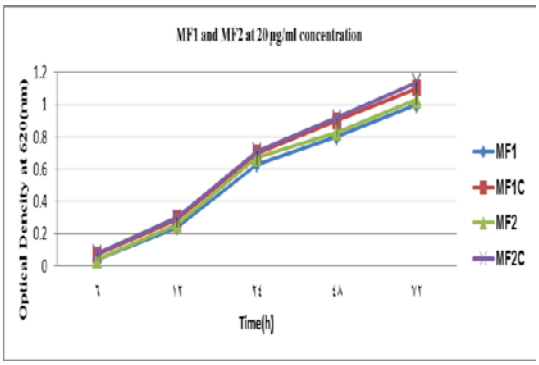
The Minimum Inhibitory Concentration (MIC) of the bacterial isolates was investigated using plate assay to select bacterial isolates capable of growing and tolerating a high level of metal toxicity. The isolates showed a very high degree of resistance to all heavy metals. MIC of cadmium was 100 to 150µg/ml for all the isolates. Minimum Inhibitory Concentration of Pb for all isolates was observed to be 160µg/ml. MIC of Hg was 60 to 80µg/ml for all isolates. MIC of Ni was 50µg/ml for MF1 and MF2, while for the other strains it was 60µg/ml. MIC of Cu was 150µg/mlfor MF4, while for the other strains it was 160µg/ml

(Table 2)). Among the heavy metals, copper and lead were less toxic, whereas nickel and mercury were found to be more toxic to all strains.

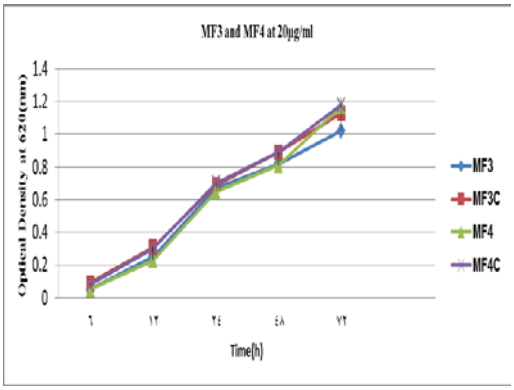
All five bacterial isolates were screened for the presence of plasmid. All the isolates except MF1 showed the presence of a mega plasmid (more than 10,000 bp) (Figure 2). To find out whether the heavy metal resistance gene was plasmid encoded or chromosomal encoded, plasmid curing was carried out by ethidium bromide. Curing results showed that though the growth of the bacteria was retarded on Cd-amended medium after plasmid curing, but it was not completely inhibited (Table 2).

Table 1. Morphological and biochemical Characteristics of the selected isolates

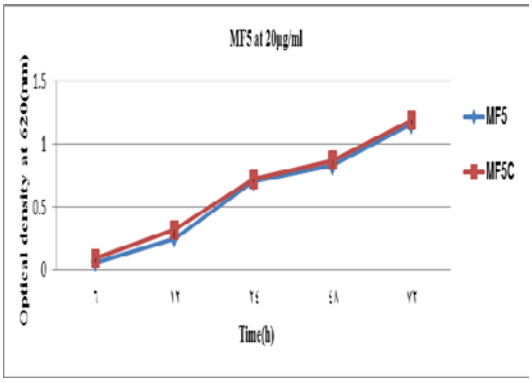
Characteristics	Bacterial isolates				
	MF1	MF2	MF3	MF4	MF5
Gram's reaction	+	+	-	-	-
Colony color	Milky white	white	white dull	Pale yellow	yellow
Spore formation	+	+	-	-	-
Starch hydrolysis	+	+	-	-	-
H_2S production	-	-	+	+	+
Catalase	+	+	-	-	-



(a)



(b)



(c)

Figure1. Growth of isolates (MF1, MF2, MF3, MF4 and MF5) in presence of Cd (20 µg/ml), C= Control

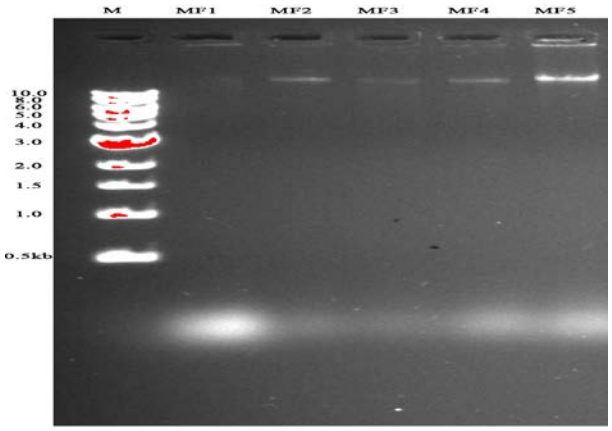


Figure2. Plasmid DNA was extracted and separated by agarose gel electrophoresis. (DNA Marker in lane1).

Table 2. Minimal inhibitory concentrations ppm) of various heavy metals before and after plasmid curing

Bacterial isolates	MIC(ppm)								
	Before Curing				After Curing				
	Cd	Pb	Hg	Cu	Cd	Pb	Hg	Cu	Ni
MF1	100	160	80	160	100	160	80	160	50
MF2	120	160	80	160	60	100	40	100	40
MF3	120	160	60	160	60	100	30	80	40
MF4	140	160	60	160	70	100	30	80	40
MF5	150	160	60	160	80	120	30	100	40

Cadmium removal assay conducted in the presence and absence of sodium thiosulfate showed that there was only 27-35% cadmium removal by isolates MF3, MF4and MF5 as compared to 75-85% by MF1 and MF2 in the absence of sodium thiosulfate. In the presence of sodium thiosulfate, MF3, MF4 and MF5 showed an increase in cadmium removal (75-80%) whereas cadmium removal remains unaffected by MF1 and MF2, after 24h of incubation. TEM analysis showed the entrapment of heavy metal in the EPS (Figure 2).Cadmium removal carried out in minimal media supplemented with 100µg/ml $CdCl_2$ showed a decrease in cadmium removal, while it remains same in isolates MF3, MF4 and MF5 when removal assays are conducted in minimal media supplemented with sodiumthiosulfate (Table 5).

Table 3. Percentage removal of cadmium by bacterial isolates from medium with initial concentration of 100µg/ml cadmium in the presence and absence of Sodium thiosulfate after 24h of incubation at 30^0C.

Bacterial isolates	LB media+100µg/ml$CdCl_2$	LBmedia+3mM $Na_2S_2O_3$+100µg/ml$CdCl_2$
MF1	85	86
MF2	75	77
MF3	29	75
MF4	33	80
MF5	27	77

Table 4 . Cadmium Removal assay under condition of Glucose deficiency.

Heavy metal(µg/ml)	Bacterial isolates				
	MF1	MF2	MF3	MF4	MF5
Cadmium	100	60	40	40	80
Lead	160	100	100	100	120
Mercury	80	40	30	30	30
Copper	160	100	80	80	100
Nickel	50	40	40	40	40

Bacterial isolates	Minimal media+$CdCl_2$		Minimal medium+$CdCl_2$+$Na_2S_2O_3$	
MF1	60	40	70	80
MF2	36	35		
MF3	100	15	70	120
MF4	30	25	77	30
MF5	80	15	62	80

Since the isolates were found to produce hydrogen sulfide, it could be one of the probable mechanisms to provide resistance to cadmium by removing the cadmium as cadmium sulfide. For the determination of cadmium sulfide production, two experiments were performed. For a simple sulfide detection assay, a semisolid agar medium containing 2.5mM $FeCl_2.4H_2O$ and 3mM $Na_2S_2O_3.5H_2O$ was used. The formation of a black precipitate (FeS) in the medium was considered to be an indication of sulfide production. Results showed in Table 4 demonstrate that Cd was efficiently recovered from the solution using bacterial produced H_2S only. The formation of black precipitate was not observed in two isolates (MF1 and MF2). Cadmium removal by bacterial isolates was also investigated in the presence of 3mM $Na_2S_2O_3.5H_2O$ and $CdCl_2$. Among the five, three isolates showed bright yellow precipitate in culture medium after 24h. Results indicate that Cd was precipitated in the form of cadmium sulphide.

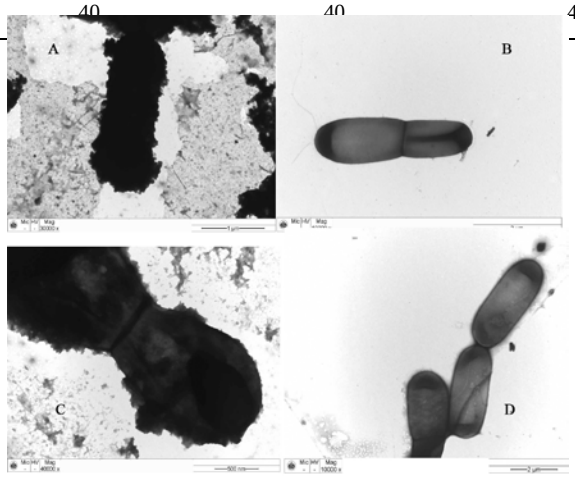


Figure 2. Transmission electron micrographs of isolates MF1 and MF2 (A: MF1grown in the presence of 10µg/ml Cd, B: MF1control, C: MF2 grown in the presence of Cd, D: MF2 control).

4. Discussion

It is very well understood that the environment continuously exposed to heavy metal contaminants favors the growth of bacteria that have developed resistance systems against heavy metal toxicity. Microbial populations in the chronically polluted sites have the capacity to degrade vast range of polluting chemicals. It has been reported that the sites subjected to chronic anthropogenic forces exhibit selection forcatabolically adaptable microbial populations other than the ubiquitous one (Bargiela *et al.*, 2015). In the present investigation, five cadmium resistant bacteria displaying multiple resistances to various heavy metals were isolated from the soils of industrial area, Faridabad. All isolates were able to grow in the presence of 20µg/ml $CdCl_2$ without any significant increase of the lag phase. Similarly results were reported earlier also by Anyanwu and Ugwn (2010).However it has also been reported that at higher concentrations bacteria undergoes physiological adjustment and shows relatively longer lag phase (Krishnamurthy and Rajaram, 2014). All isolates were also found to be resistant to various heavy metals to varying degrees as depicted by their MIC values. The resistance to heavy metals in bacterial strains has been reported to be conferred upon through various mechanisms (Ron *et al.*, 1992). Heavy metals may enter the cell as an alternative substrate for cellular ions transport system. While some bacteria employ mechanisms that cause changes in the transport systems so that the heavy metals no longer enter the cell, others possess several ATPase dependent efflux mechanisms that confer resistance (Tynecka *et al.*, 2016). Precipitation on the cell surface in the form of $CdHPO_4$ and binding of Cd^{2+} by thiols is another mechanism reported in many bacteria (Sinha and Mukharjee, 2009). In another study, Cadmium has been shown to bind to capsular material in *Arthrobacter viscosus* and in *Klebsiella aerogenes* (Hrynkiewicz *et al.*, 2015). A *Citrobacter* mutant isolated from metal-polluted soil was found to accumulate Cd^{2+} as insoluble cell-bound $CdHPO_4$ during growth in the presence of Cd^{2+} and glycerol (Macaskie *et al.*, 1987).

The present work indicates that the isolated bacterial isolates have the ability to resist a wide range of heavy metals. It was observed that all bacterial isolates have multiple heavy metal tolerance and are resistant to Cd, Ni, Pb, Cu and Hg. Resistance to multiple metals has been found in several other bacterial systems and characterized at the molecular level. Liesegang *et al.* (1993) reported that *AlcaligeneseutrophusCH34* harbors numerous heavy metal resistance determinants including three for mercury resistance, one for chromate resistance and two for divalent cations, called czc (for Cd^{2+}, $Zn^{2+}$and Co^{2+}) and cnr (for $Co^{2+}$and Ni^{2+}). Pandit *et al.* (2013) reported that metal resistant bacterial isolates showed high degree of resistance to heavy metals ranging from 25-300ppm. Singh *et al.* (2010) studied that *Pseudomonas aeruginosa* exhibited high resistance to heavy metals with MIC for heavy metals ranging from 50µg/ml to 300µg/ml.

CadA and *cadB* operons represent the two known mechanisms of plasmid-mediated cadmium resistance widespread in bacteria. Bacterial plasmids have genes that confer highly specific resistance to As, Bi, Cd, Cu, Cr, Hg, Zn and other toxic heavy metals. For each toxic cation and anion, generally a different resistance system exists, and these systems may be linked together on multiple resistance plasmids (Silver *et al.*, 1989). In the present study, the plasmid profile of the isolates was found to exhibit a single band indicating the presence of a mega plasmid (more than 10,000bp). Results indicate the presence of plasmid in the selected isolates except MF1. It has been found that large plasmids are responsible for encoding resistance to antibiotics and heavy metals (Jain *et al.*, 2009). Resistance to heavy metals by genes present on their plasmid suggests the extraction of selective pressure on such bacteria through contamination with heavy metals in their environment. Plasmid curing inhibited the growth of all isolates except MF1on media containing cadmium reflecting that the mechanisms involved in conferring resistance are both plasmid as well as chromosomal mediated. Studies have revealed the existence of chromosomal determinants that mediate heavy metal resistance in many organisms. Cad A gene has been found to be a chromosomal determinant in gram positive like *Bacillus subtilis* (Solovieva and Entian, 2004), *Bacillus firmis* (Oger *et al.*, 2003) and gram negative like *Stenotrophomonas maltophilia* (Alonso *et al.*, 2000). Cadmium removal assays showed that there was only 27-33% cadmium removal by isolates MF3, MF4, MF5 as compared to 75-85% byMF1, MF2, reflecting the presence of an active efflux mechanism encoded by cad A operon which although plays an important role in conferring the resistance but is not helpful in the removal of cadmium. They are able to grow in the presence of cadmium but not able to detoxify it. In case of isolatesMF1 and MF2, it was observed that detoxification is achieved by entrapment in the extracellular polymeric substance as determined by TEM. To ascertain the same, the cadmium removal studies were also done under the conditions of glucose limitation under which the production of EPS is greatly inhibited. It was observed that cadmium removal was significantly inhibited in the strains that remove cadmium through EPS and not in strains that precipitate cadmium as cadmium sulfide indicating that biosorption of heavy metals through EPS is one of the mechanisms employed by the isolate used in the present study. Various bacteria have been implicated in removal of heavy metals from industrial wastes and soil through functional groups on their cell envelops (Volesky, 1986; Brierly, 1990). Bacterial extracellular polymeric substances play an important role in metal sorption and constitute a passive method in which the metal cations bind to the negative charges of acidic groups from expolysaccharide.

Since the isolatesMF3, MF4 and MF5 were found to produce hydrogen sulfide, it may also be a putative mechanism for the removal of cadmium in the form of cadmium sulfide. To confirm the formation of cadmium sulfide, the cultures were grown in the broth containing 50mM cadmium chloride and after 24h of incubation a bright yellow colored precipitate was formed due to cadmium sulfide precipitation. Cadmium removal assay was conducted in the medium containing thiosulfate and

it showed 75-86% cadmium removal indicating that one of the mechanisms of resistance to heavy metals is through the production of hydrogen sulfide (H_2S). This production of sulfide might confer cadmium resistance in these isolates for its survival under cadmium stress and it detoxifies cadmium by converting it into insoluble CdS. Many soluble metals can form insoluble complexes with hydroxides, carbonates, phosphates, and sulfides (Gadd and Griffiths, 1978; Fortin *et al.*, 1997). One of the best known natural metal precipitation mechanisms is due to sulfide production H_2S and is produced when sulphur-containing amino acids are decomposed (Valls and Lorenzo, 2002) Microorganisms secrete inorganic metabolic products such as sulphide ions in their respiratory metabolism and with them precipitate toxic metal ions as a form of non-enzymatic detoxification. Metal sulfides possess low solubilities and, therefore, low toxicities because they are biologically unavailable. Many studies have been undertaken with the aim of determining the mechanism of biotransformation of cadmium into cadmium sulfide. *Klebsiealla planticola (Cd-1)* grew anaerobically at a Cd concentration of 15mM and precipitated CdS (Sharma *et al.*, 2000). Bang *et al.* (2002) developed a genetically engineered bacterium capable of producing sulfide under aerobic, microaerobic, or anaerobic conditions for heavy metal precipitation. Microbial population inhabiting polluted sites may have ability to resist much higher concentrations may employ a variety of mechanisms to detoxify the same. As such, efforts need to be directed in revealing and exploiting their real potential. It has been reported that the biodegradation is a process which is mostly performed by the autochthonous bacteria and if environmental condition are optimized using an efficient *ex-situ* treatment such as land farming, such indigenous populations will likely out perform any allochthonous consortium (Fordelianakis *et al.*, 2015). As such, the application of metal-resistant bacteria isolated from the contaminated site for bioremediation offers attractive perspectives. In addition, this approach may also prove to be useful in biological treatment of other organic wastes through protection rendered by the metal accumulating strains to the organic matter degrading bacteria.

5. Conclusion

In the present study, the cadmium resistant bacterial isolates were isolated from the soils collected from Faridabad industrial area. These isolates were found to be resistant to a number of heavy metals besides cadmium. The mechanism involved in conferring resistance to heavy metals was found to be both chromosomal as well as plasmid mediated. Cadmium removal is found to be through both metabolism independent (entrapment in extracellular matrix) and metabolism dependent mechanism (cadmium sulfide production).The detoxification efficiency indicates good potential for application in bioremediation of cadmium from polluted sites. According to the present study, it is very clear that any bacteria that show a fairly good resistance to heavy metals and are capable of producing EPS are potential candidates for heavy metals removal from contaminated site.

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Heavy Metals, Nutrients, Total Hydrocarbons and Zooplankton Community Structure of Osse River, Edo State, Nigeria

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Abstract

An aquatic ecological survey was carried out across Osse River from April, 2013 to September, 2014. The study was aimed at assessing the heavy metals, nutrients and total hydrocarbons in the water and sediment in conjunction with the zooplankton biodiversity. Surface water, sediment and zooplankton samples were collected from four (4) selected stations. Station 1 was chosen as control station upstream, far away from perturbations; while Stations 2, 3 and 4 were chosen at locations of distinct anthropogenic activities. Surface water and bottom sediment samples were analyzed for heavy metals, nutrients and total hydrocarbons using Atomic Absorption Spectrophotometer (AAS) and Gas Chromatograph-Flame Ionization Detector (GC-FID), respectively. Zooplankton specimens were sorted and dissected where necessary under a binocular dissecting microscope (American Optical Corporation, Model 570), while counting and identifications were done with an Olympus Vanox Research Microscope (mag X60) Model 230485. Results showed that the river is an oligotrophic aquatic ecosystem. The significant Varimax rotated matrices of manganese (0.947), copper (0.883) and zinc (0.817) revealed that these parameters were the active components in the water; while that of copper (0.896) was the active component in the sediment. This revealed that essential metals were the active components in both media. This is consistent with the fact that concentrations of manganese in the water at Stations 3 (0.97 mg/l) and Station 4 (1.26 mg/l), and copper at Station 4 (1.05 mg/l) slightly exceeded the regulatory limits. The zooplankton individuals were spatially distributed in the following order: Station 1 (923) > Station 4 (385) > Station 3 (191) > Station 2 (123). The lowest number of zooplankton individuals were recorded at Stations 2 and 3, i.e., locations of highest perturbations where high concentrations of manganese, nickel and THC were recorded in the water, and nickel, lead, copper and THC were recorded in the sediment. The high concentrations of manganese and THC; particularly Ni which was higher than other stations and FEPA limit, can be attributed to oil exploration activities, such as gas flaring, petrochemical production, storage and transit. Perturbation in zooplankton community structure is prognostic of possible impacts on other aquatic biota of economic relevance. There are indications that anthropogenic activities at Osse River are liable to cause severe ecological perturbations in future if not put in constant check. Continuous stringent bio-monitoring study of the aquatic environment is recommended to put the levels of heavy metals, nutrients and total hydrocarbons in constant check.

Keywords: Zooplankton, surface water, sediment, heavy metals, nutrients, total hydrocarbons.

1. Introduction

Osse River is a major source of water, finfish and shellfish for the populace within communities in the watershed. However, incessant perturbations due to anthropogenic activities are potential threats to the aquatic biota which hold substantial economic values. The activities include oil exploration and exploitation, agricultural practices, discharge of domestic and industrial wastes, laundering and logging. These pollutants are released into the aquatic environments through different pathways, such as point source discharges, surface run-offs, leaching and atmospheric deposition. These activities are capable of disrupting the delicate aquatic ecological equilibrium. Unfortunately, water and sediment are receptors of anthropogenic chemicals as well as habitats to aquatic organisms.

Variability in water and sediment properties is a function of a number of factors which have been reported in previous studies by numerous authors. Generally, these factors can be categorized as authoctonous and allothonous factors working in tandem. Ogbeibu and Victor (1989) reported that perturbations from road and bridge construction across Ikpoba River, Benin City, Nigeria, had a significant impact on the sediment which in turn had impacts on the vital benthic invertebrates. Benka-Coker and Ohiomian (1995) reported on the significant effect of slaughter house waste on the water and sediment qualities of Ikpoba River and warned against threats to the aquatic fauna which are of nutritional relevance to the dependent populace.

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Tukura *et al*. (2012) attributed variation of physico-chemical properties of water and sediment of Mada River, Nasarawa State, Nigeria to seasonal variation, i.e., higher concentrations of most parameters observed in dry season was attributed to increase in concentration as a result of reduced water volume in the dry season.

The water matrix of an aquatic ecosystem is the first receptor of the contaminants released from anthropogenic activities. The sediment of the river eventually serves as repository to these contaminants (Adams *et al.*, 1992; Camusso *et al.*, 1995). However, the rate of deposition of these contaminants is a function of the sorption capacity, which varies among contaminants. Ogbeibu *et al*. (2014) observed that manganese, zinc, copper, cadmium, lead and total hydrocarbons had very high sorption capacities from water into the sediment of Ikpoba River. They therefore strictly recommended biomonitoring of the parameters.

The distinct anthropogenic activities at Osse River are capable of releasing toxicants into the aquatic environment (water and sediment). These toxicants can be readily accumulated by the fauna and flora through processes of bioconcentration, bioaccumulation and biomagnification (Isibor and Oluowo, 2016). Toxicants rise to significant concentrations as they are transferred from one trophic level to the higher, up the pyramid of biomasses through food chain. This might ultimately culminate in public health concerns.

Zooplanktons are a unique group which are suitable bio-indicators in biomonitoring studies. This is due to their unique position in the food chain; as the primary consumers and their high sensitivity to physico-chemical alterations in their ambience. Several researchers have sought to use zooplanktons as bio-indicators of aquatic perturbations. Innumerable studies have been carried on zooplankton using the water quality as the basic background reference. Some detailed zooplankton study in the Niger Delta areas of Nigeria include Imoobe and Adeyinka (2010), Ezekiel *et al* (2011), Ogbuagu and Ayoade (2012), Iloba and Ruejoma (2014), Mandu and Imaobong (2015) to mention a few. However, no existing holistic study has been done on water, sediment and zooplankton biodiversity; with a view to providing the picture of the entire aquatic environment at a glance. Therefore the study was aimed at assessing the heavy metals, nutrients and total hydrocarbons in the water and sediment; in conjunction with the zooplankton biodiversity.

2. Material and Methods

2.1. The Study Area

The research was conducted on a stretch of Osse River, which traverses Nikorowa, through Ekehuan and Gelegele and terminates at Iziedema community. It lies between latitude 5° 90' - 6° 60' N and longitude 5° 18' - 5° 23' E (Figure 1). It is a lotic freshwater with a thick vegetation canopy along its bank. The predominant vegetation around the river includes palm trees (*Elaeis guinensis*), shrubs, floating *Salvinia* species, *Lemna*s pecies and water hyacinth (*Eichorrnia crassipes*). The river is located in the Ovia North- East Local GovernmentArea, Edo State, within the tropical rainforest belt, in the southern part of Nigeria. Water flows in south-westerly direction into the river from Akpata Hills in Ekiti State. It then flows further downstream through the Gwato creeks; into the Benin River, which empties into the Atlantic Ocean. For the purpose of the current study, four Four (4) stations were chosen along the stretch of the river based on distinct anthropogenic activities. Station 1 (control station) was upstream, located at Nikorowa upstream, far away from perturbations, while Stations 2 was located at (Ekehuan, about 4,135 metres downstream from of Station 1), Station 3 (Gelegele, 4, 441 metres downstream from Station 2), and Station 4 (Iziedema, 1, 400 metres downstream from Station 3) were chosen at locations of distinct anthropogenic activities. At the Ekehuan section (Station2) of the river, innumerable drums of crude oil were stored at the bank of the river. An oil company named Dubri Oil Company carries out oil exploration activities at the Gelegele section (Station 3) of the river. Constant gas flaring was also observed at this section. Immense lumbering activities were observed at the bank of Iziedema section (Station 4). These activities are potential perturbation sources to the aquatic environment.

Figure 1. Map of the study area

2.2. Collection and Analysis of Samples

The samples were collected monthly from April 2013 to September 2014 at all the stations. Surface water samples were collected in 250 ml glass containers with lid and properly labelled. Sediment samples were collected using a Birge-Ekman grab. The sediment samples were collected in foil papers and wrapped with labelled polythene. Qualitative plankton samples were collected by towing a 55 µm mesh hydrobios plankton net tied to a 25 HP engine-powered boat driven at about 2 knots just below the water surface for 5 minutes. Quantitative samples on the other hand were collected by filtering 100 liters of water fetched with a bucket through a 55 µm mesh hydrobios net. Both samples were preserved separately in 4% buffered formalin solution. All samples were preserved in ice coolers and transported immediately to the laboratory for analysis. For quality control and standardization measures, these laboratory procedures were repeated at least 3 times and mean values were compared with standards set by FEPA (2003).

2.2.1. Analysis of Water and Sediment

2.2.1.1. Heavy Metals and Nutrients

Water samples were pretreated and digested using the wet oxidation method (Martin *et al*., 1992). The varian Techron spectra AA- 10 Atomic Absorption Spectrometer (Serial No. 9021318) with an attached printer was used for the qualitative determination of heavy metals and nutrients. The sample was fixed with 2 ml of 0.05 M Nitric acid (Martin *et al*., 1992). The mixture was filtered through Whatman filter paper number 1 and aspirated directly into the Atomic Absorption Spectrometer (AAS) for metals and nutrients determination, having prepared the blanks accordingly. For qualitative assurance purposes the purpose the AAS was calibrated for each parameter by dissolving 1 gram analar grade of each element in 1 liter of distilled water. Standard and corresponding blanks were run with each set of experimental digest to ascertain quality control. The results of the analysis were cross checked using standards set by FEPA (2003).

1 gram air-dry sediment sample was placed in a 300-mlL calibrated digestion tube. 3ml concentrated nitric acid (HNO3) was added, swirled carefully and placed in a rack to settle. The mixture was slowly heat up by gradually increasing temperature to about 145 °C for 1 hour (Estefan *et al*. 2013). 4 ml concentrated Perchloric acid (HClO4) was added and heated to 240°Cfor another 1 hour. Mixture was allowed to cool to room temperature. It was filtered through Whatman No. 42 filter paper and the volume was made up to 50 ml with de-ionized water. Heavy metals were then determined by Atomic Absorption Spectrophotometer (model-analyst 200 PerkinElmer).

2.2.1.2. Total Hydrocarbons (THC)

50 ml of water sample was collected in a conical flask. 20 ml of dichloromethane was also added into the flask. The flask was shaken and pressure released at intervals. The sample was allowed to stand for few minutes. Consequently, two layers were formed in the flask. The lower layer (extract) of the sample was collected into a beaker through a filter paper. The filtrate was concentrated to 1 ml by evaporation at room temperature overnight in a fume cupboard (LAWI, 2011).

10g of air-dried sediment sample was added into an amber glass bottle. 20g of anhydrous sodium sulfate (Na2SO4) was also added into the glass bottle containing the soil sample and stirred to remove moisture from the sample. 300 µg/ml of surrogate (1-chlorooctadecane) standard was added to the soil sample. 30 ml of dichloromethane (extracting solvent) was added to the sample and the bottle was corked. The bottle containing the mixture was agitated for about 6 hours at room temperature using a mechanical shaker (LAWI, 2011). After agitation, the sample was allowed to settle for 1 hour and then filtered through 110 mm filter paper into a clean beaker. The filtrate was allowed to concentrate to 1 ml by evaporation overnight in a fume cupboard.

The separation and detection of compounds in sediment and water samples were carried out using Agilent 6890N Gas Chromatograph-Flame Ionization Detector (GC-FID) instrument according to LAWI (2011), which was slightly modified by Cortes *et al*. (2012). 3 µl of concentrated sample was injected into Gas Chromatography (GC) vial. The blank dichloromethane was injected into micro-syringe of GC to clean the syringe (3 times) before taking the sample for analysis. The micro-syringe was further rinsed with the sample. Then, the sample was injected into the column for separation of compounds in the sample. After separation the compounds were passed through a Flame Ionization Detector (FID). FID detected the compounds in the sample. The amount of total hydrocarbons was ascertained at a particular chromatogram in mg/kg for sediment samples and in mg/l for water samples.

2.2.2. Analysis of zooplankton

In the laboratory, specimens were sorted and dissected where necessary under a binocular dissecting microscope (American Optical Corporation, Model 570), while counting and identifications were done with an Olympus Vanox Research Microscope (magX60) Model 230485. Identification of specimens was carried out at the University of Benin, Zooplankton laboratory using identification keys provided by Van de Velde (1984), Jeje and Fernando (1986) and Boxshall and Braide (1991).

2.2.3. Statistical Computations

In order to discern the major parameters of key importance, i.e., responsible for alterations in the environmental matrices analyzed, the principal components of the water and sediment samples were analyzed using descriptive statistics such as communalities, total variance, percentage variance and rotated component matrix. Parameters with communality values less than 0.75 were considered insignificant while components with Eigen values less than 1 were also considered insignificant; hence eliminated so that fewer components were further subjected to the Varimax rotation stage using Keiser normalization method. The descriptive statistics such as the mean, range and standard error were for significant differences in the heavy metals, nutrients and total hydrocarbons in water and sediment samples was done using ANOVA ($P < 0.05$). Duncan Multiple Range (DMR) test was used to identify the source of variance.

The percentage relative abundance of the zooplankton was estimated by direct count. Each quantitative sample was concentrated to 10 ml and1 ml of sample was taken and all individual taxa present were counted. Relative abundance was calculated as the number of individuals per 100litres.The diversity of the zooplankton was expressed using biodiversity indices such as taxa Richness (R), Evenness (E), Dominance (D) and Shannon-Weiner diversity (H), which were computed using Paleontological Statistics Software (PAST). The sorption capacities of heavy metals and THC were assessed using the Distribution co-efficient (Kd).

$$(Kd) = \frac{M_{ads}}{M_{sol}}$$ (Soares and Alleoni, 2006)

; where M_{ads} = metals adsorbed into the soil and M_{sol} = metal concentration in water.

3. Results and Discussion

3.1. Heavy Metal, Nutrients and THC in Water and Sediment

3.1.1. Water

In the water, spatially heterogeneous patterns of some of the parameters analyzed were apparent in the result. As shown in Table 1, concentrations of iron in water of Stations 2, 3 and 4 were much significantly higher than that of Station 1 ($P < 0.001$). The levels of iron though slightly above the control station were however within FEPA (2003) acceptable limit for aquaculture. The concentrations of manganese and lead in the water of Stations 3 and 4 were significantly higher than Stations 1 and 2 ($P < 0.001$). Omoigberale and Ikponmwosa- Eweka (2010) also reported that the level of manganese in water at Gelegele (Station 3) was higher than limit within the period of July, 2000 to June, 2002. Oguzie and Ehigiator (2011) observed a reduction in the level of manganese from July to September, 2007 at same location below the acceptable limits. The periodical variability in the levels of manganese can be at-tributed to varying anthropogenic activities. At Station 2; the location of most severe crude oil activities and illegal operations, the concentrations of nickel and total hydrocarbons (THC) in water were very much significantly higher than other stations and even established standard limits. Table 2 show high spatial heterogeneity in metal, nutrients and THC loads in sediment across all stations. This evidence of repository nature of sediment was earlier reported by Camusso et al. (1995).

Table 1. Summary of heavy metal, nutrients and total hydrocarbons (in mg/l) in water of Osse River

PARAMETERS	**STATION 1** **MEAN±S.E(RANGE)**	**STATION 2** **MEAN±S.E(RANGE)**	**STATION 3** **MEAN±S.E(RANGE)**	**STATION 4** **MEAN±S.E(RANGE)**	**P value**	FEPA (2003)
Fe	**0.45 ± 0.16^{B} (0 – 2.4)**	**1.71 ± 0.25^{A} (0 – 3.5)**	**1.44 ± 0.19^{A} (0 – 2.9)**	**1.38 ± 0.27^{A}(0.2 – 5.4)**	**P<0.001**	20
Mn	**0.02 ± 0.01^{B} (0 – 0.1)**	**0.24 ± 0.06^{B} (0 – 0.7)**	**0.97 ± 0.22^{A} (0 – 2.3)**	**1.26 ± 0.34^{A} (0 – 3.7)**	**P<0.001**	0.5
Ni	**0.004 ± 0.03^{C}(0 – 0.4)**	**2.59 ± 0.15^{A} (0.1 – 2.6)**	**3.92 ± 0.3^{B}(0.2 – 5.2)**	**0.09 ± 0.25^{C}(0 – 3.3)**	**P<0.05**	1
Pb	**0.001 ± 0.003^{B}(0 – 0.1)**	**0.08 ± 0.01^{A}(0 – 0.2)**	**0.83 ± 0.24^{A} (0 – 2.7)**	**0.03 ± 0.26^{A} (0 – 2.7)**	**P<0.001**	< 1
Cu	**0.02 ± 0.01^{B}(0 – 0.1)**	**0.14 ± 0.03^{B} (0 – 0.5)**	**0.13 ± 0.02^{B} (0 – 0.4)**	**2.05 ± 0.26^{A} (0 – 2.76)**	**P<0.001**	< 1
SO_4	**2.5 ± 0.4^{B}(0 – 5.2)**	**5.4 ± 1.1^{A}(0.1 – 13.5)**	**3.5 ± 0.5^{B}(0.1 – 7.5)**	**2.2 ± 2.8^{B}(0.1 – 5)**	**P<0.01**	-
NO3	**0.68 ± 0.18^{C}(0 – 2.1)**	**2.67 ± 0.53^{A}(0 – 6.5)**	**1.93 ± 0.24^{B}(0 – 3.4)**	**1.36 ± 0.33^{B}(0 – 3.1)**	**P<0.001**	20
PO_4	2.17±0.14(1.5 – 3.4)	2.57±0.28(0.5 – 4.5)	2.15±0.19(0.7 – 3.9)	1.74±0.21(0.6 – 3.3)	P>0.05	<5
THC	**0.02 ± 0.01^{D} (0 – 0.1)**	**6.19 ± 0.6^{A} (0 – 10.5)**	**4.77 ± 0.2^{B} (0- 1.89)**	**1.26 ± 0.28^{C} (0 – 3.2)**	**P<0.001**	10

Note: Values with similar superscripts indicate no significant difference. Number of samples= 18. P>0.05 impliesthere is no significant difference, **P<0.05** means there is significant difference, **P<0.01** means there is much significant difference, and **P<0.001** means there is very much significant difference

3.1.2. Sediment

The levels of iron in the sediment at Stations 2, 3 and 4 were very much higher than that of the control station and FEPA limit for aquatic aquaculture. Concentration of copper was also higher in the water at Station 4 than other stations including regulatory limit. The levels of primary productivity nutrients (sulfate, nitrate and phosphate) show that Osse River is an oligotrophic aquatic ecosystem. This agrees with the findings of Imoobe and Adeyinka (2010). Concentrations of copper and total hydrocarbons were also higher in the sediment of other stations than the control station. High concentrations of manganese, nickel, copper and THC observed in the water may result in chronic sub-lethal effects and de-creased biodiversity of the biota in the water column.

Manganese could cause nervous system disruptions in finfish and shellfish, which may result in inefficiency in escape from predators and search for food and mates; and ultimately reduced biodiversity (Isibor *et al*., 2016). At the highest trophic level, manganese concentrations may rise through the processes of bio-magnification and could ultimately elicit neurological disorders similar to Parkinson's disease in man (ATSDR, 2005). High concentrations of some heavy metals in the water and sediment of the perturbed locations may cause severe ecological disruptions in Osse River. THC in the water and sediment may elicit teratogenic, carcinogenic, mutagenic and immunosuppressive effects both in biota and man (ATSDR, 2010). Relatively higher concentrations of nickel, lead and THC observed in the water and sediment at Station 2 can be attributed to the reckless crude oil handling which was prominent at this station. Relatively higher concentrations of iron, nickel, lead and total hydrocarbons; particularly in the sediment samples of Stations 3 and can be attributed to the significant sorption capacities of the metals (Table 3). Figures 2 and 3 further showed at a glance that most of the parameters analyzed were deposited in the sediment. These deposited pollutants can be released back into the water column; causing perpetual rise in the aqueous phase.

Table 2. Summary of heavy metal, nutrients and total hydrocarbons (in mg/kg) in sediment of Osse River

	STATION 1	STATION 2	STATION 3	STATION 4		FEPA (2003)
PARAMETERS	MEAN±S.E(RANGE)	MEAN±S.E(RANGE)	MEAN±S.E(RANGE)	MEAN±S.E(RANGE)	P value	
Fe	0.3 ± 0.06^{D}(0- 0.9)	1.91 ± 0.2^{A}(0.9- 3.8)	2.28 ± 0.2^{B}(0.6- 3.4)	1.4 ± 0.5^{C}(0.4- 3.4)	P<0.001	1
Mn	0.1 ± 0.05^{D}(0- 0.9)	1.01 ± 0.21^{B}(0- 2.6)	5.67 ± 0.23^{A}(0.1- 4.2)	0.97 ± 0.36^{C}(0.2- 2.4)	P<0.001	0.4
Ni	0.35 ± 0.06^{D}(0- 0.9)	19.58 ± 0.28^{A}(0.2- 4.1)	6.19 ± 0.19^{B}(0.7- 3.2)	1.1 ± 0.3^{C}(0.1- 2.3)	P<0.001	-
Pb	0.01 ± 0.001^{C}(0- 0.03)	6.04 ± 0.01^{A}(0- 0.08)	2.04 ± 0.01^{B}(0- 0.2)	0.04 ± 0.08^{C}(0- 0.1)	P<0.001	0.05
Cu	0.05 ± 0.015^{C}(0- 0.2)	2.72 ± 0.248^{A}(0- 3.6)	2.16 ± 0.219^{A}(0- 2.6)	0.56 ± 0.37^{B}(0.1- 1.3)	P<0.001	0.3
SO_4	0.49 ± 0.08^{C}(0.1 – 1.7)	2.27 ± 0.356^{A}(1.1- 6.1)	2.11 ± 0.17^{A}(1.3- 3.4)	1.86 ± 0.58^{B}(0.5- 3.2)	P<0.001	240
NO_3	1.41 ± 0.17^{C}(0.4 – 2.7)	3 ± 0.27^{A}(1.7- 5.2)	3.49 ± 0.18^{A}(2.3- 4.6)	2.88 ± 0.1^{B}(1.8- 4.4)	P<0.001	40
PO_4	2.05 ± 0.17^{D}(1.1 – 3.6)	3.54 ± 0.23^{C}(2.3- 5.5)	5.07 ± 0.31^{A}(3.1- 7.8)	4.07 ± 0.82^{B}(2.5- 6.7)	P<0.001	5
THC	0.24 ± 0.05^{D}(0- 0.6)	68.435 ± 0.165^{A}(0.3- 2.4)	14.15 ± 0.52^{B}(0.4-8.3)	2.09 ± 0.3^{C}(0.3- 3.8)	P<0.001	-

Note: Values with similar superscripts indicate no significant difference. Number of samples= 18. P>0.05 means there is no significant difference, **P<0.05** means there is significant difference, **P<0.01** means there is much significant difference, and **P<0.001** means there is very much significant difference

Table 3. Distribution co-efficient of heavy metals, nutrients and total hydrocarbons

Fe	Mn	Ni	Pb	Cu	SO_4	NO_3	PO_4	THC
11.18	3.13	**24.61**	**13.41**	2.66	1.33	2.59	1.7	**66.51**

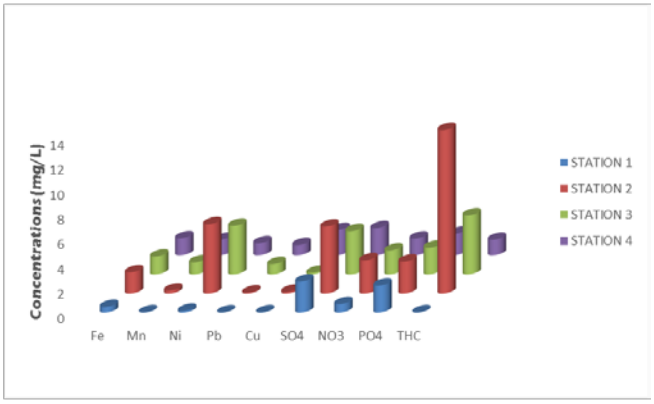

Figure 2. Concentrations of heavy metals, nutrients and THC in water

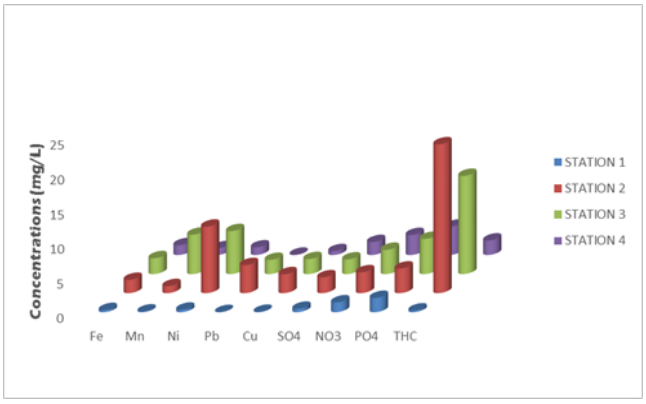

Figure 3. Concentrations of heavy metals, nutrients and THC in sediment

3.2. Zooplankton Community Structure

The relative percentage composition of the taxonomic groups recorded during the study period was Rotifera (41.12%) > Copepoda (30.64%) > Cladocera (20.72%) > Calanoida (7.52 %).This conforms to the trend observed at Ekpan River by Iloba and Ruejoma (2014). Rotifers (represented by 13 taxa) were the most represented among all the groups observed. The group was dominated by *Conochilus unicornis* which constituted 88 individuals.

Cladocerans were also well represented by 10 taxa in the zooplankton community with *Ilvocryptus spinifer* having the highest number of individuals (53). Copepoda was represented by 9 taxa and it was dominated by *Microcyclops varicans*; having 69 individuals. The least represented was Calanoida, which had 3 taxa, dominated by *Thermodiaptomus galebi*; 44 individuals. The presence of tropical Freshwater species such *Synchaeta longipes* and *Conochilus dossuarius*, coupled with the absence of *Pompholyx sulcata, Proales sp., Keratella tropica, Keratella quadrata*, *Bronchionus anguilaris*, and *Trichocera pusilla* indicates an oligotrophic to mesotrophic aquatic system. This can be attributed to the moderate concentrations of nitrate, phosphate and sulfate observed in the water of the aquatic environment throughout the period of study (Imoobe and Adeyinka, 2010). Furthermore, the absence of *Diaptomus minutus* at Stations 2 and 3 can be linked to high concentrations of manganese, nickel and THC (Mohammed, 2006).

The zooplankton individuals were spatially distributed in the following order: Station 1 (923) > Station 4 (385) > Station 3 (191) > Station 2 (123). The lowest number of zooplankton individuals were recorded at Stations 2 and 3, i.e., locations of highest perturbations where high concentrations of manganese, nickel and THC were recorded in the water (Table 1), and nickel, lead, copper and THC were recorded in the sediment (Table 2). The high concentrations of manganese and THC; particularly Ni which were higher than other stations and FEPA limit (Table 1) can be attributed to oil exploration activities such as gas flaring, petrochemical production, storage and transit. Mortality of zooplankton due to perturbations from anthropogenic activities has been reported in many literatures. Almeda *et al*. (2013) reported the mortality of innumerable zooplankton species due to exposure to crude oil.

The Taxa Richness (R) and Shannon-Wienner Diversity (D) of zooplankton at Station 2 and 3 were significantly lower than that of Station 4, which was higher than that of Station 1 (Table 5). The Copepoda group comprises of individuals with adaptive resilience to oil-associated environmental stressors. Of the four groups at Stations 2 and 3 Copepods have an outstanding number (Table 4); particularly *Thermocyclops neglectus* which dominated Station 2 (18 individuals) and Station 3 (16 individuals). Other Copepods which dominate the impacted stations include *Afrocyclops curticonis, Diacyclops thomasi, Ectocyclops phaleratus, Eucyclops agiloides, Halicyclops korodiensis, Mesocyclops minutues, and Micocyclops varicans* (Table 4). The dominance of the Copepods is reflected in the relatively high Dominance Indices at Station 2 (1.02) and Station 3 (0.98), coupled with the relatively low Taxa Richness (R) which are 1.14 and 1.89 respectively (Table 5). The percentage distribution of zooplanktons is in the order of Station 1 (57%) > Station 4 (24%) Station 3 (12%) > Station 2 (7%). The significantly lower numbers of individuals at Stations 2 and 3, compared to Stations 1 and 4 are additional evidences of significant spatial impacts of anthropogenic activities.

Table 4. Species composition and percentage occurrence of Osse River Zooplankton. Sample size (N) = 18

SPECIES COMPOSITION	STATION 1	STATON 2	STATION 3	STATION 4	TOTAL	% OCCURRENCE
Cladocera						
Alona rectangula	28	1	1	12	42	2.65
Bomina longirostris	18	0	2	11	31	1.96
Bosminopsis deitersi	22	9	2	12	45	2.84
Ceriodaphnia cornuta	28	0	1	4	33	2.08
Chydorus sphaericus	18	1	1	6	27	1.71
Diaphanosoma excisum	21	0	1	3	25	1.58
Echinisca triseralis	24	2	2	4	32	2.02
Ilyocryptus spinifer	42	1	3	7	53	3.35
Kurzia longirostris	19	0	1	4	24	1.52
Macrothrix spinosa	14	0	0	2	16	1.01
Copepoda						
Afrocyclop curticornis	22	3	1	14	40	2.53
Diacyclops thomasi	12	4	1	12	29	1.83
Ectocyclops phaleratus	18	11	12	8	49	3.10
Eucyclops agiloides	22	8	18	14	62	3.92
Halicyclops korodiensis	23	13	19	12	67	4.23
Mesocyclops leukarti	28	4	22	11	65	4.11
Metacyclops minutus	21	8	13	3	45	2.84
Microcyclops varicans	28	14	15	12	69	4.36
Thermocyclops neglectus	15	18	16	10	59	3.73

Calanoida						
Diaptomus minutus	22	0	0	12	34	2.15
Thermodiaptomus galebi	26	2	6	10	44	2.78
Tropodiaptomus incognitus	18	1	8	14	41	2.59
Rotifera						
Ascomorpha ovalis	17	3	9	12	41	2.59
Asplanchna priodonta	19	2	4	8	33	2.08
Brachionus diversicornis	12	0	3	6	21	1.33
Collotheca sp	10	1	2	6	19	1.22
Conochilus dossuarius	56	0	1	8	65	4.11
Conochilus unicornis	68	0	2	18	88	5.56
Euchlanis dilatata	71	0	6	15	52	3.28
Kellicottia longispina	42	1	0	28	71	4.49
Keratella cochlearis cochlearis	32	4	0	16	52	3.28
Keratella longispina	28	3	8	14	53	3.28
Synchaeta longipes	31	9	7	19	66	4.17
Trichocerca cylindrica chattoni	26	0	4	21	51	3.22
Trichocerca similis	22	0	0	17	39	2.46
TOTAL=	**923**	**123**	**191**	**385**	**1583**	**100**

Table 5. Biodiversity of the zooplankton community of Osse River between April, 2013 and September, 2014

Descriptive Indices	STATION 1	STATION 2	STATION 3	STATION 4	P VALUE
No. of Species	35	23	30	35	P>0.05
No. of Taxa	4	4	4	4	P>0.05
No. of Individuals	923^{A}	123^{C}	191^{C}	385^{B}	P<0.001
Taxa Richness (R)	6.32^{A}	1.14^{B}	1.89^{B}	5.87^{A}	P<0.05
S. Wienner Diversity (D)	3.32^{A}	0.24^{B}	0.45^{B}	3.42^{A}	P<0.05
Eveness (E)	0.876^{A}	0.132^{B}	0.334^{B}	0.89^{A}	P<0.01
Dominance Index (C)	0.75^{B}	1.02^{A}	0.98^{A}	0.45^{C}	P<0.05

Note: Values with similar superscripts indicate no significant difference. Number of sample replicates = 18. P>0.05 means there is no significant difference, **P<0.05** means there is significant difference, **P<0.01** means there is much significant difference, and **P<0.001** means there is very much significant difference

4. Conclusion

The present study showed a detailed proactive investigation of suspected anthropogenic disruptions using the zooplankton community as a predictive tool. Anthropogenic activities had a significant impact on the community structure of the zooplankton. This necessitates further detailed research to ascertain the possible ecological and public health risks nickel, copper, manganese and total hydrocarbons may pose. Impacts on zooplankton community structure are prognostic of possible impacts on other aquatic biota of economic relevance. We recommend a continuous stringent bio-monitoring study of the aquatic environment to put the levels of heavy metal, nutrients and total hydrocarbons in constant check.

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Species Identification Based on trnH-psbA and ITS2 Genes and Analysis of Mineral Nutrients of Selected Medicinal Plants from Malaysia

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Abstract

Accurate plant identification and screening of the elements in medicinal plants have become increasingly important. Some elements can become toxic when existing at high concentrations. The present study presents a molecular identification method to discriminate plant species and assess the mineral nutrients in four selected medicinal plants widely used in traditional healing practices in Malaysia. Medicinal plants were sampled from a secondary forest in Northern Peninsular of Malaysia. Species discrimination was conducted using phylogenetic inference and BLAST method on two target genes, trnH-psbA and ITS2 while the concentration levels of the mineral in terms of macro and micro nutrients (Ca, Fe, Mg, Mn, Zn, Cu) were determined using ICP-OES spectroscopy in the leaf samples. The present study revealed that the DNA identification method has successfully discriminated all samples to species level and that the trnH-psbA is the best marker for identification. The concentration levels of mineral nutrients ranged from 0.01 to 5.76 mg/kg. within the safety range as recommended by the World Health Organization (WHO). Results of the present study provide important data on the DNA barcoding of medicinal plants and assessment of mineral nutrients, which can be useful for providing scientific information on dietary supplements based on authentic medicinal plants and products.

Keywords: DNA barcoding, trnH-psbA, ITS, micronutrient, macronutrient, medicinal plants, ICP-OES.

1. Introduction

Existing taxonomic identification of plants group is mainly morphological based. However, there are limitations to relying primarily on morphology when attempting to identify plants species during various stages of their development or when examining fragmentary or processed remains. It has been recognized that rapidly evolving mitochondrial genes, punctuated with highly conserved regions can be recovered via Polymerase Chain Reaction (Mullis, 1990). Folmer *et al.* (1994) and Hebert *et al.* (2003) demonstrated that the 5′end region of cytochrome c oxidase sub unit I (COI) is highly appropriate for discriminating between closely related species across diverse phyla in the animal kingdom, establishing it as the "DNA barcode" locus for broadly identifying animals (Ward *et al.*, 2005). However, the use of COI as a universal plant barcode does not indicate any successful story due to the generally low rate of nucleotide substitution in plants mitochondrial genomes (Hollingsworth *et al.*, 2011). Additionally, the structure of mitochondrial genome in plants has changed rapidly, thus the existence of a universal intergenic spacer at the species level will be precluded (Kress *et al.*, 2005). The Internal Transcribed Spacer (ITS) region of the nuclear ribosomal cistron is the most commonly sequenced locus for plant molecular systematic investigations at the species level (Kress *et al.*, 2005). Several chloroplast gene regions, for example, maturase K (matK) and ribulose 1,5-biphosphate carboxylase /oxygenase large sub unit (rbcL) were also widely used and considered as a core barcodes for plants (Schori and Showalter, 2011).

The important roles of medicinal plants in traditional healing systems have been documented in the literature, and it was found that most of the developing world continues to rely on this for primary health care and home remedies. In Malaysia, medicinal plants, in the form of packaged herbal prepared and manufactured by small and medium-sized industry, as well as pharmaceutical industry, are widely used (Ahmad and Othman, 2015). Consequently, Malaysian Government has chosen herbs industry as the first Entry Point Project (EPP1) for the nation's Agriculture New key Economic Area with the aim to produce

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high-value products amounting MYR 2.2 billion of the Gross National Income (Ahmad and Othman, 2015). Nowadays, in view of scientific interest, consumer demands promote the development of dietary supplements and new drugs based on medicinal plants. The use of these medicinal plant-based products is rapidly spreading in industrialised health care sector of the 21st century (Abe *et al.*, 2013; Kayani *et al.*, 2014; Siew *et al.*, 2014). Although this has been proven to improve the economy of a country, it has raised an alarming concern related to the purity of the raw plants material used in a preparation of the products. There were opportunities for substitution or adulteration of the raw ingredients of the herbal products due to misidentification of plants, lack of cultivation and the long supply chain from harvesting site to market (Schori and Showalter, 2011). Using barcoding technique, the raw material used to produce herbal products can be ascertained because the substitution within certain plant families (especially Apiaceae and Solanaceae) would give a very bad effect and could be fatal. Therefore, the accurate identification of medicinal plants in relation to their purity and quality as well as a safe application has become increasingly important (Pang and Chen, 2014).

Apart from the problems that will arise due to misidentification of plants, herbal products can be contaminated during growth, development and processing influenced by their environmental factors (Barthwal *et al.*, 2008). The medicinal values of these plants lie in their chemical substances in terms of mineral nutrients and metallic elements that are involved in physiological processes and are important for the proper functioning and maintaining good health in the human body (Gupta *et al*., 2010; Subramanian *et al.*, 2012a). These elements play a pivotal role in biochemical processes and enzyme systems in the human organism even at threshold levels (Mahmood *et al.*, 2013). For instance, calcium (Ca), chromium (Cr), copper (Cu), magnesium (Mg) and zinc (Zn) play important roles in neurochemical transmission and serve as elements of biological molecules in various metabolic processes (Okatch *et al.*, 2012).

Although these elements are often essential for living organism, they become toxic when present at high concentrations (Okem *et al.*, 2014). Street (2012) stated that the amount of mineral nutrients and heavy metals in medicinal plants are known to pose potential threats to animals and human beings that consume them or their derived products. For instance, some elements, such as iron (Fe) and magnesium (Mg), can cause ill effects when consumed at higher concentration levels (Subramanian *et al.*, 2012b). Thus, to avoid harmful effects, screening of the mineral nutrient content of medicinal plants is highly essential. According to the World Health Organization (WHO, 1998), determination of these elements in medicinal plants is a part of quality control to establish their purity, safety and efficacy. In this regard, they recommend that medicinal plants, which form the raw materials for the finished products, must be checked for the presence of heavy metals and the level of mineral nutrients and further they regulate maximum permissible limits of these elements.

Many efforts have been made to analyze the mineral nutrient contents of medicinal plants from various parts of the world by applying several techniques, such as atomic absorption spectroscopy (Virgilio *et al*., 2012; Gupta *et al.*, 2014; Dghaim *et al.*, 2015), atomic emission spectrometry (Jia *et al*., 2011; Kwon *et al*., 2014), x-ray fluorescence (Desideri *et al.*, 2010), and inductively coupled plasma mass spectrometry (Tokalıoğlu, 2012; Bu *et al*., 2013). Even though many attempts have been reported for determination of mineral nutrient of medicinal plants from all over the world (Ajasa *et al*., 2007; Street *et al.*, 2007; Street *et al.*, 2008; Erna *et al.*, 2014; Rajan *et al.*, 2014); documented reports of medicinal plants growing in Malaysia are scarce (Ong and Norzalina, 1999). The present study was designed (1) to barcode four selected medicinal plants (*Murraya koenigii, Strobilanthes crispa, Justicia gendarussa* and *Centella asiatica*), commonly used in Malaysia and (2) to analyze six mineral nutrients (Ca, Fe, Mg, Mn, Zn, Cu) that could be found in the selected medicinal plants. Most of the traditional healers in Malaysia often prescribe mixtures of these medicinal plants in the raw form for diseases ranging from diarrhea; respiratory system and cancer; skin disease, malaria; high cholesterol level and high blood pressure (Duñg and Loi, 1991; Brinkhaus *et al.*, 2000; Sikder *et al.*, 2011; Gul *et al.*, 2012; Kadir *et al.*, 2014).

2. Material and Methods

2.1. Samples Collection

Ten samples of medicinal plants were randomly collected from June to September 2013 from an eight-acre secondary forest located in Northern Peninsular of Malaysia (Table 1). Whole plant and/or parts of the plant were cut and collected with sharp scissors. The collected plant materials were then put in a sterile polyethylene bag and labeled before transported straight to the Molecular Ecology Laboratory, School of Biological Sciences, Universiti Sains Malaysia. Morphological identification of the plants collected was conducted with the help of local taxonomist from the School of Biological Sciences, Universiti Sains Malaysia and a book titled Photographic Atlas of Botany and Guide to Plant Identification authored by Castner (2005) (Table 1). The voucher samples were deposited in the herbarium of Universiti Sains Malaysia. Based on a search for the literature on the most common medicinal plants used by people in Peninsular Malaysia, only four plant samples were included for further analysis.

2.2. DNA Extraction, Amplification and Sequencing

Approximately 200 mg fresh young leaves were ground to a fine paste and homogenized in a DNA extraction buffer [(50 mM Tris HCL pH 8.0, 25 mM EDTA pH 8.0, 150 mM NaCl, 40.0 ml H2O) and 1 g PVP 40 mw 40 000]. The mixture was made up to 100 ml with distilled water and the pH was adjusted to pH 5.0 with HCl. The ground material was then transferred to a 2 ml tube. The modification of the cetyl trimethylammonium bromide (CTAB) protocol by Cota- Sánchez (2006) was used for DNA isolation procedure of the leaf materials. Briefly, 500 µl of CTAB buffer was added into 2 ml tube contained ground material. The mixture of plant extract and CTAB buffer was then incubated overnight at 56°C and mixed intermittently by inversion. Subsequently, 250 µl of chloroform was added to the mixture and then mixed again by inversion. The tube was then spun at 12000 g for 5 minutes. Two layers of aqueous phase were formed and only the upper aqueous phase that contains

DNA was transferred to a new sterile tube. The DNA was precipitated with 50 µl 7.5M ammonium acetate (NaAc, pH 4.6) and two volumes of 95% EtOH before incubated at -20 0C for 1 hour.

The tube was centrifuged at 13000 rpm for 15 minutes to isolate the precipitates. The supernatant was removed and the DNA pellet was washed twice by adding 500 µl ice-cold 70% EtOH. After the tube was centrifuged for 10 minutes, the ethanol was poured off, and then the tube was centrifuged again for a few seconds before removing the remaining liquid using a pipette. The tube was then left to dry in the incubator (with the cap open) for 30 min, or until the remaining liquid had evaporated. Then, DNA pellet was then resuspended in Milli-Q water depended on the amount of isolated DNA. DNA was then incubated at 65°C for 20 minutes to destroy any DNases that may have been present and stored at -20°C. To obtain a high-quality DNA, free from polysaccharides and other metabolites that might interfere during PCR amplification, purified DNA concentration of each sample was estimated by ethidium bromide-stained band intensities against λ DNA.

Polymerase Chain Reaction (PCR) was used to amplify the target region of the gene in the nuclear or plastid genome of plants depending on the primer pairs used. The primers used in the present study are ITS2-2F, 5'-GCGATACTTGGTGTGAAT-'3, ITS2-R, 5'-GACGCTTCTCCAG ACTACAAT-3', psbA-F, 5'-GTTATGCATGAACGTAATGCTC-'3 (Sang *et al.*, 1997) and trnH-R, 5-'CGCGCATGGATTCACAAATC-'3 (Sang *et al.*, 1997). The PCR reaction consisted of 10X PCR reaction buffer, 25 mM MgCl2, 1.25 mM of each dNTPs, 1 unit of Taq polymerase, 10 µM of each primer, 20 ng genomic DNA and 30 µl Milli-Q water. Thermal cycling conditions (on a T100TM Thermal Cycler; BioRad, Singapore) were 35 x [94°C for 45 s, 40°C - 50°C (depends on the primer used) for 45 s, 72°C for 1 min] and a final incubation at 72°C for 10 min. Additional purification was performed on all samples using the Qiagen DNA Mini Kit (Germany) to remove contaminants, such as pigments, tannins and other polymerase chain reaction inhibitors). Purified PCR products were then sent to First Base Laboratories Sdn Bhd (1st BASE) for sequencing. At 1st BASE, DNA sequencing was performed using a BigDye® v3.1 Terminator (Applied Biosystems) sequencing kit, with approximately 20-30 ng of cleaned PCR products and 1.6 ρmol of primer (forward and reverse separately) in each reaction. Sequencing products were cleaned and then electrophoresed on an ABI 3100xl capillary sequencer following standard protocols.

2.3. Sequence Analysis and Species Identification

The amplified partial of ITS2 and trnH-psbA region of collected medicinal plants was used in the basic local alignment search tool (BLAST) algorithm with the ITS2 and trnH-psbA gene of the same species using BLASTn from GenBank (available at http://blast.ncbi.nlm.nih.gov). In addition, a total of 83 (trnH-psbA) and 67 (ITS2) conspecific sequences of the collected plant samples were retrieved from GenBank. All sequences were collapsed in haplotypes using the Collapse software version 1.2 (Provan *et al.*, 2005). Haplotypes were then aligned using Clustal W version 2.0.12 (Larkin *et al.*, 2007) in combination with a total of 150 sequences of trnH-psbA and ITS2 retrieved from the GenBank database. All sequences were manually checked and trimmed in the Bioedit version 7.2.5 sequence editing program (Hall, 1999); alignments were then subsequently revised by eye in an effort to maximise positional homology. All positions containing gaps and missing data were eliminated from the data sets.

Species discrimination was evaluated according to tree-based analysis in which the Neighbour Joining (NJ) tree was adopted and performed in MEGA version 5.0 (Tamura *et al.*, 2011) with a K2P molecular evolutionary model and branch supports were determined using 10,000 bootstrap replicates. Successful identification using this method was inferred when sequences from the same species formed a monophyletic group.

The DNA barcoding gaps which defined as the spacer region between intra and inter specific genetic variations and identification efficiency was also implemented to investigate the breaks in the distribution of genetic pairwise distances and performed in Automatic Barcode Gap Discovery (ABGD) (Puillandre *et al.*, 2012). This method proposes a standard definition of the barcode gap and can be used to partition the data set into candidate species even when two distributions overlap (see Liu *et al.*, 2014). Sequences of trnH-psbA and ITS2 data set were uploaded to http: //www.abi.snv.jussieu.fr/public/abgd/abgdweb.html. For this analysis, we set up values for the prior P (prior maximum divergence of intraspecific diversity) ranging from 0.001 to 0.1 as if the P value is set too high, the whole data set will be considered as a single species (Puillandre *et al.*, 2012). The distance analysis was calculated based on a K2P analysis.

2.4. Sample Preparation for Analysis of Mineral Contents

Successfully identified plant species (based on DNA characterization; see section above) were used in this analysis. Leaf samples were washed with fresh running water to remove dirt, dust and other contaminated agents and afterwards the leaf samples were re-washed with deionized water for more cleaning. They were dried in a shade at room temperature (22-25°C). The dried samples were crushed, powdered and homogenized using an agate mortar and pestle. The powdered samples were kept in polyethylene sampling bags separately until analyzed.

2.4.1. Sample Digestion

The microwave digestion of leaf samples was done in accordance with US EPA Method 3052. Dried and powdered of leaf sample of 0.5 g each was weighed directly into the digestion vessel liners. Nine mL of concentrated HNO_3, 0.5 mL of concentrated HF, 0.5 mL HCl and 1.0 mL of H_2O_2 were added to each vessel. The vessels were allowed to react for approximately one minute prior to sealing the vessel. Then, the vessels were sealed, placed in a rotor and heated in a microwave system for 20 minutes. The temperature profile was specified to permit specific reactions and incorporates reaching 190±5 ºC in approximately less than 5.5 minutes and remaining at 190±5 ºC for 9.5 minutes for the completion of reactions. After cooling, the vessel contents were filtered into a 100 mL volumetric flask and diluted with deionized water.

2.4.2. Analytical Methods

Mineral nutrients (Ca, Fe, Mg, Mn, Zn, and Cu) in the selected medicinal plant samples were analysed using Perkin Elmer Optima 7000 DV ICP-OES instrument equipped with WinLab32 for ICP Version 4.0 software. Sample introduction system was checked and absorption wavelength for the determination of each metal together with its linear working range was tuned. Three replications and sample sensitivity according to sample concentration were then selected. Sample sensitivity according to sample concentration was then selected. The concentration values were taken from the average of three reading. These samples can be directly introduced to the ICP-OES along with standards that were prepared earlier. The working standards used were 1, 2, 4 and 8 mg/L. The standards were prepared by serial volume/volume dilution in polypropylene vials. Standards preparation from stock solution was set based on the equation: $V_1 = M_2V_2 / M_1$, where, V_1, V_2, M_1 and M_2 represent: volume taken from stock solution (mL), volume of DI water (20 mL), concentration of stock solution (ppm) and concentration of working standard (ppm), respectively.

PerkinElmer NIST traceable calibration standards for ICP were used as the stock standards for preparing working standards. Certified reference standards for 18 elements in plant samples were used for validating the developed method. Multi-element standards (Merck, Germany) for ICP for 18 elements in 65% nitric acid (HNO3) were used as quality control check standards. As for digestion purpose, 49% HNO3, 37% hydrochloric acid (HCl), 30% hydrogen peroxide (H2O2) and 49% hydrofluoric acid (HF) were used.

3. Results and Discussion

3.1. Sequencing Success

A total of 158 sequences for two barcodes were analysed, from which 8 sequences (for both trnH-psbA and ITS2 genes) were successfully obtained from the collected medicinal plants and 150 sequences were obtained from GenBank. Each sample was successfully amplified by the 530 bp (ITS2) and 542 bp (trnH-psbA) fragments. In comparison, the amplification reactions were performed with full success (100%) for trnH-psbA, whereas ITS2 demonstrated lower reaction efficiency with successful amplification of 95% of samples. Several attempts per sample were needed for ITS2 adjustment of the PCR reagents (i.e., DNA template, dNTPs, Taq DNA Polymerase), which demonstrated significant variability in amplification success among different plant samples. High quality sequences were obtained for the amplified DNA samples in which trnH-psbA showed the highest efficiency (100% sequencing success) and ITS2 showed only 75% success.

The internal transcribed spacer of nuclear ribosomal DNA (nrDNA ITS) has been used as a universal barcode in discrimination of more than 6600 plant samples (Chen *et al.*, 2010; Liu *et al.*, 2012). In the present study, the ITS2 region showed lower sequencing success when compared to trnH-psbA region. Similar results were also reported in other studies in which the sequencing success was considered low (see e.g., Sass *et al.*, 2007; Hollingsworth, 2011; Tripathi *et al.*, 2013). Difficulty in amplifying and sequence has been identified as the main limitation for ITS (Hollingsworth *et al.*, 2011). This drawback thus has been used as an argument for considering ITS as less acceptable as a standard DNA barcode in some research (Bolson *et al.*, 2015).

3.2. Performance of Markers in Species Discrimination

BLASTn analysis for all sequences showed that of the four samples analysed, trnH-psbA was correctly identified 100% of the sequences both at species and genus level, respectively, indicating that the scientific names recovered from the BLASTn analysis matched the putative scientific names expected based on vernacular names and morphological identification (Table 1). Likewise, trnH-psbA gene consistently shows the highest success rate in species and genus identification (99.8%) when BLASTn analysis was employed for samples from wide range taxa. In contrast, the correct identification for ITS2 was lower both at the genus and species level which only 75% for the four collected samples and only 69.5% for samples retrieved from GenBank. Based on these results, the present study reveals that trnH-psbA is the most reliable DNA marker for the medicinal plants collected from Peninsular Malaysia (Table 1).

The variation of names used for the same medicinal plants throughout different races, ethnics and geographic range of the species is likely the most important reason for the mismatch in species identification by BLASTn and the corresponding plant species that based only on their vernacular names (Mankga *et al*., 2013). For example, in Malaysia, the name Pecah beling is mostly referred as *Baphicacanthus cusia*, *Strobilanthes crispus* or *Saricocalix crispus*. Other than Pecah Beling, the plant is also called Pecah kaca or Jin batu while in Indonesia it was called Pecah beling, Enyoh kilo, Kecibeling or Kejibeling. A study by Abu Bakar *et al.* (2006) showed that *Strobilanthes crispus* exhibit high antioxidant activity as well as anticancer properties by inhibiting the proliferation of cancer cell lines in vitro. Moreover, consumption of food products contained *S. crispus*, for example, tea could contribute to the additional antioxidant needed in the body to enhance defence system, as well as an additional nutraceutical supplement in patients diagnosed with breast cancer (Abu Bakar *et al*., 2006). However, inaccurate identification of plants will compromise the therapeutic value of medicinal plants, thus would endanger human health. In Iran, the case of *Bunium cylindricum* (adulterant) that was mixed with Zire-e-siah (*Bunium persicum*) and sold in the market has resulted in the degradation of the quality and efficacy of the drug (Joharchi and Amiri, 2012). Many cases of toxicity have been reported and mostly due to species misidentification (Viljoen, 2013). The situation, where a proliferation of herbal remedies that have been adulterated or substituted with other plant materials, has stressed the need for a quality control (Raterta, 2014).

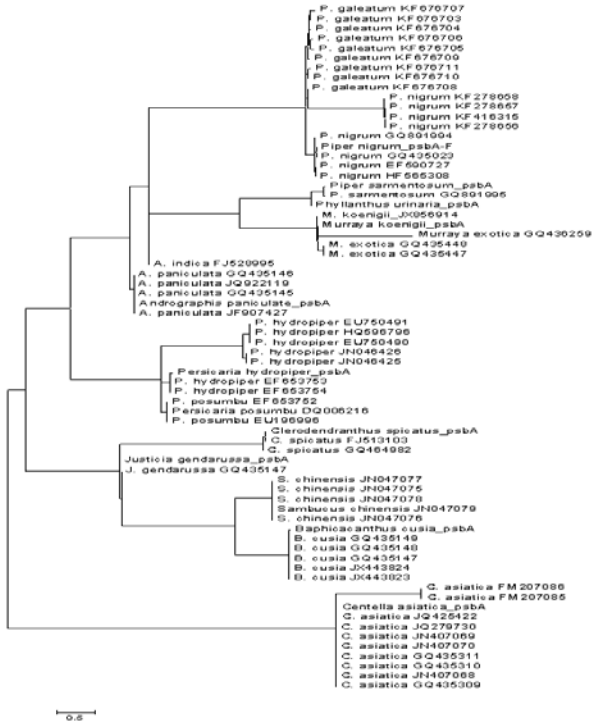
Table 1. List of medicinal plants analyses and BLAST analysis with the percentage of maximum identity for each sample

No	Local name	DNA region			
		ITS2		trnH-psbA	
		Scientific name	Max Id (%)	Scientific name	Max Id (%)
1	Kari	*Murraya koenigii*	99	*Murraya koenigii*	99
2	Pecah Beling	*Sambucus chinensis*	91	*Baphicacanthus cusia*	99
3	Ganda Rusa	*Justicia gendarussa*	99	*Justicia gendarussa*	97
4	Pegaga	*Centella asiatica*	98	*Centella asiatica*	99

3.3. Species Discrimination Based on Phylogenetic Trees

An ideal DNA barcode must have adequate conserved regions for universal primer design, enough variability to be used for species identification (CBOL) and have a high ability to differentiate between closely related species, which will be only achieved when the genetic distance between species is significantly higher than within congeneric species (Hebert *et al.*, 2004; Mankga *et al.*, 2013). Barcoding studies normally use phylogenetic trees to assign species names and the most commonly utilized tree is neighbor joining, in which the assessment was based on phenetic distance and evolutionary information of a species (Liu *et al.*, 2014). Based on the neighbor-joining tree as shown in Figure 1, each barcode marker used was successfully separated each genus by representing a monophyletic clade, in which each clade appeared distinctly distant from other clades. For example, the clade containing genus Piper (*P. galeatum* and *P. nigrum*) and Murraya (*M. koenigii* and *M. exotica*) as displayed in *trnH-psbA* (Figure 1a) and ITS2 (Figure 1b) phylogenetic trees was well supported. The trnH-psbA genes demonstrated full successful identification (100%) both at genus and species level. However, the identification success of ITS2 barcode is only 98.7%. In this case, the positions of some species are ambiguous in which they were grouped within a different species and/or genus. For example, based on ITS2 gene, *Sambucus chinensis* (sequence obtained from this study) was largely separated from its origin where this species was located within the species *Baphicacanthus cusia* (sequence retrieved from GenBank) (Figure 1b).

Molecular systematic approaches have traditionally relied on comparing a limited number of the orthologous sequence to obtain estimates of species relationships across the tree of life (Edger *et al.*, 2014). Additionally, an assumption of phylogenetic analysis of nucleotide sequences is that each position is independent of other positions was always made in analysing phylogenetics relationship of a species (Alvarez and Wendel, 2003). The results of the present study demonstrated that the majority of sequence sites for ITS2 are not independently evolving, but rather are co-evolving with at least one other position in order to preserve the secondary structure of a molecule. Thus, in future studies, effort should be made to identify all co-evolving sites and appropriate adjustments are needed before employing ITS2 region as a phylogenetic marker.



(a)

(b)

Figure 1. Neighbour joining tree of selected medicinal plants collected from North Peninsular Malaysia and conspecific sequences retrieved from GenBank for (a) trnH-psbA *and* (b) ITS2. Sequences with red arrow show the species with ambiguous lineage.

3.4. Composition of Mineral Nutrients

The mean concentration levels of mineral nutrients in the four selected medicinal plants are summarized in Table 2.

3.4.1. Calcium (Ca) and Magnesium (Mg)

The concentration level of calcium (Ca) is highest in *Strobilanthes crispa* with 5.76 mg/kg followed by *Centella asiatica* (5.71 mg/kg), *Justicia gendarussa* (5.26 mg/kg) and *Murraya koenigii* with 5.16 mg/kg (Table 2). High concentration of Ca is essential for all organisms due to its role in blood coagulation and necessary for proper functioning of the muscle and nervous systems (Straub, 2007). Meanwhile, the concentration levels of magnesium (Mg) in *M. koenigii*, *S. crispa*, *J. gendarussa* and *C. asiatica* were 1.37 mg/kg, 1.13 mg/kg, 1.72 mg/kg and 1.49 mg/kg, respectively.

Mg is a constituent of bones, teeth and enzyme cofactor in which it will be absorbed in the intestines and then transported through the blood to cells and tissues (Soetan *et al.*, 2010). The present study revealed that all plants analyses provide very low source of Mg as the necessary daily intake of Mg is 350 mg/day for men and 300 mg/day for women (WHO, 1998). Magnesium depletion will result in chronic or excessive vomiting and diarrhoea and acute magnesium deficiency result in vasodilatation, in which erythemia and hyperaemia will appeare a few days on the deficient diet (Champagne, 2008; Soetan *et al.*, 2010).

Table 2. Concentration levels of mineral nutrients (mg/kg) in the selected medicinal plants.

	Plants			
Element (mg/kg)	Kari *Murraya koenigii*	Pecah Beling *Strobilanthes crispa*	Ganda Rusa *Justicia gendarussa*	Pegaga *Centella asiatica*
Ca	5.16 ± 0.0	5.76 ± 0.0	5.26 ± 0.0	5.71 ± 0.0
Cu	0.01 ± 0.0	0.01 ± 0.0	0.01 ± 0.0	0.01 ± 0.0
Fe	0.15 ± 0.0	0.14 ± 0.0	0.15 ± 0.0	0.21 ± 0.0
Mg	1.37 ± 0.02	1.13 ± 0.0	1.72 ± 0.0	1.49 ± 0.02
Mn	0.0 ± 0.0	0.± 0.	0.± 0.02	0.± 0.
Mo	0.0 ± 0.	ND	0.01 ± 0.	0.01 ± 0.
Zn	0.02 ± 0.	0.03 ± 0.	0.03 ± 0.	0.01 ± 0.

3.4.2. Iron (Fe) and Manganese (Mn)

Among the selected medicinal plants, the mean concentration level of iron (Fe) varied between 0.14 and 0.21 mg/kg. Fe is important for the formation of haemoglobin and plays an essential role oxygen and electron transfer in a human body (Omokehide *et al.*, 2013). In cellular respiration, it functions as an important component of enzymes involved in biological oxidation (Soetan *et al.*, 2010). In the present study, the highest concentration level of Fe was found in *C. asiatica* (0.21 mg/kg) while *S. crispa* had the lowest concentration (0.14 mg/kg) (Table 2). WHO has recommended an intake of Fe of 10-20 mg/day for an adult and the permissible level of Fe in medicinal plants is 20 mg/kg (WHO, 1998). Even though WHO has formulated the guideline for quality assurance, the lack of knowledge and heavy use of medicinal plants may contribute to tissues damage and some diseases in humans. In contrast, deficiency of Fe has been reported to have a role in brain development and in the pathophysiology of restless leg's syndrome (Soetan *et al.*, 2010).

Manganese (Mn) concentration level can be found highest in *J. gendarussa* with mean concentration 0.37 mg/kg, while the lowest concentration was in *S. crispa* with mean concentration 0.04 mg/kg. *Murraya koenigii* and *C. asiatica* share the same concentration as presented in Table 2. Mn is an important element as a structural component of some enzymes as stated by (Saracoglu *et al.*, 2001). Mn is an essential trace element for the growth of plants and animals. Specifically, it is important in normal reproductive functions and normal functioning of the central nervous system (Devi and Sarma, 2013). Its deficiency produces severe skeletal and reproductive abnormalities in mammals. High concentration of Mn causes hazardous effects on lungs and brains of human (Son *et al.*, 2007). The recommended intake level for Mn is 11 mg/day for an adult and the maximum permissible limit of Mn in medicinal plants is 200 mg/kg (WHO, 1998).

3.4.3. Zinc (Zn) and Copper (Cu)

Justicia gendarussa and *S. crispa* contained the highest zinc (Zn) concentration level of 0.03 mg/kg followed by *M. koenigii* (0.02 mg/kg) and *C. asiatica* (0.01 mg/kg) (Table 2). Zinc is essential to all organisms and has an important role in metabolism, growth, development and general well-being, as well as become a crucial co-factor for numerous enzymes in the body (Omokehide *et al.*, 2013). Zinc deficiency, particularly in children can lead to loss of appetite, growth retardation, weakness and even stagnation of sexual growth (Daur, 2015). Recommended limit of Zn in medicinal plants reported in WHO (1998) is 50 mg/kg, while its intake in food is 11 mg/day (Khan *et al.*, 2008). Thus, the concentration level of Zn observed in the present study was within the safe limit. While the concentration of Zn in the selected medicinal plants in the present study shows various ranges, the concentration of copper (Cu) was found in the same level in all four plants, which is 0.01 mg/kg (Table 2). Copper is an essential redox-active transition element that plays vital role in various metabolic processes and forms a component in many enzyme systems in the human body (Omokehide *et al.*, 2013). Due to the toxic properties of Cu, its quantity in plants should be very low. As reported by Negi et *al.* (2012), a high concentration of Cu causes metal fumes fever, hair and skin decolorations, dermatitis, respiratory tract diseases and some other fatal diseases in human beings. WHO permissible limit of Cu in medicinal plants is 10mg/kg, while its intake in food is 2-3mg/day (WHO, 1998).

4. Conclusion

Four medicinal plants from Peninsular Malaysia (*Murraya koenigii*, *Strobilanthes crispa*, *Justicia gendarussa* and *Centella asiatica*) were successfully identified based on DNA characterization at two target genes namely trnH-psbA and ITS2. Using the BLAST analysis and genetic distance method as inferred by phylogenetic tree, the present study suggests that

trnH-psbA is the best marker for identification of medicinal plants from Northern Peninsular Malaysia. The concentration levels of mineral nutrients (Ca, Fe, Mg, Mn, Zn, and Cu) in leaf samples of the selected medicinal plants were successfully determined using ICP-OES techniques. The observed concentration levels of the elements in the leaf samples of identified medicinal plants were found within the trace concentrations and significantly below the permissible limits. This indicates that the leaves of all medicinal plants in the present study are safe to use and consume. Further research on the use of other target regions for species discrimination and analysis of mineral concentration levels in other tissues of these medicinal plants would help to establish baseline data on these species.

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Bacterial and Fungal Communities Associated with the Production of A Nigerian Fermented Beverage, "Otika"

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Abstract

"Otika" is a Nigerian indigenous alcoholic beverage produced from sorghum. The present work investigates the microbial community and exhibition of mutualism or antagonistic interaction during the production of 'Otika'. The microbes were isolated, enumerated and identified by pour plate, streak, morphological and biochemical characterization methods. Microbial interactions between the isolates identified were investigated by Agar well assay technique. Total bacterial, fungal, lactic acid bacterial and enterobacteriaceae counts, respectively, increased from $1.6\times 10^6 \pm 0.33$ cfu//ml, $3.4 \times 10^5 \pm 0.10$ cfu/ml, $3.0 \times 10^6 \pm 0.0$ cfu/ml and $1.5\times 10^6 \pm 0.15$ cfu/ml to $4.6 \times 10^7 \pm 0.30$ cfu/ml, $4.5 \times 10^6 \pm 0.10$ cfu/ml, $9.0 \times 10^7 \pm 0.05$ cfu/ml and $3.7 \times 10^7 \pm 0.2$ cfu/ml on the sorghum grains and at early stages of fermentation. Later, bacterial load decreased steadily along the fermentation period while enterobacteriaceae decreased until it was undetectable. *Bacillus* species, *Staphylococcus aureus*, *Enterobacter cloacae*, *Escherichia coli*, *Lactobacillus plantarum*, *Lactobacillus fermentum*, *Pediococcus acidilactici*, *Enterococcus faecalis*, *Leuconostoc mesenteroides*, *Saccharomyces cerevisiae*, *Saccharomyces* species, *Candida krusei*, *Candida tropicalis*, *Aspergillus* species and *Penicilium italicum* were identified. Microbial occurrence through the production stage ranged from 33.3% each for *E. cloacae*, *A. fumigatus* and *Penicilium italicum* to 100% each for *L. plantarum*, *S. cerevisiae* and *C. tropicalis.* Yeasts and lactic acid bacteria exhibited positive interaction. There were no antagonistic interactions that existed among *L. plantarum*, *L. fermentum*, *Leuconostoc mesenteroides* and *S. cerevisiae* whereas both were antagonistic against other bacteria. The present study sheds more light on the populations and types of bacteria and fungi with their associations that characterized the production of "Otika" which will be useful information for production of consistent quality "Otika".

Key Words: "Otika" a Nigerian beverage, Microbial communities, Microbial interactions, Sorghum.

1. Introduction

Indigenous traditional beverage plays a vital role in the daily social, economic, nutritional and cultural aspects of people's life especially in developing countries (Kadjogbé *et al*., 2015; Fowoyo and Ogunbanwo, 2016). "Otika" is brownish-opaque, sweet with slightly sour taste (Ogunbanwo and Ogunsanya, 2012). It is an indigenous alcoholic beverage produced originally from sorghum grains through traditional process involving indigenous fermentation technology (Achi, 2005; Ogunbawo and Ogunsanya, 2012). "Otika" is commonly made in the South-Western Region of Nigeria "Otika" is used for various traditions including hospitality, friendliness and as part of the etiquette of most families. "Otika" also serves to seal harmonious relationships between individuals (Solange *et al*., 2014).

Fermentation is the process whereby chemical transformations of organic substances are broken down into simpler compounds by the actions of enzymes (Rina and Sonali, 2016). It has many advantageous attributes, which include improving nutritional value and safety of foods against pathogens over non-fermented foods (Adebayo *et al.*, 2014). Fermentation contributes to the reduction of some secondary metabolites, such as tannins and polyphenols in addition to enhancing the taste, aroma, shelf life, texture, nutritional value and other attractive properties of foods (Nzigamasabo and Nimpagaritse, 2009; Embashu, 2014; Stephanie *et al*., 2015). Fermented foods form about 25% of the foods consumed worldwide (Adebayo *et al.*, 2014).

Wide spectrum of microorganisms is involved in the production processes of fermented foods but a few types usually determine the quality of the end products. In order to access the types of microbes involved in determining the quality of "Otika", the present work was designed to investigate the microbial flora and exhibition of mutualism and antagonistic interaction during the production of "Otika." Therefore, isolation, characterization and identification of the microorganisms involved in the production with a prospective selection of starter cultures that are adapted to "Otika" production would be important to support the

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technical process and obtain a predictable end product with desired quality.

2. Materials and Methods

Traditional Preparation of "Otika"

Sorghum grains and water were mixed in ratio 1:3 (w/v) and left for two days at 25±2°C to steep. Malting followed in moistened fresh banana leaves for 3 days at 25±2°C. The germinated grains were dried at ambient temperature of 25±2°C for 2 days and milled using grinding instrument. Wort was extracted from the milled malted sorghum grains by cooking (100°C) for 7 hours, allowed to cool, poured into earthenware pots and left to naturally ferment for 3 days at 25±2°C (Ogunbanwo and Ogunsanya, 2012).

Sample Collection of "Otika"

Sample of traditionally fermented "Otika" were obtained at each step of production (Sorghum to the 120hrs of fermentation) from local home-made "Otika" producer (This production site is a major location where other retailers patronize from) in 'Iseyin', Oyo State, Nigeria. It was collected three different times from the period of March to May, 2012. These samples were put in sterilized bottles and transported to the Department of Microbiology's Laboratory, Federal University of Technology Akure for microbial analysis.

Microbial Analysis

Enumeration and isolation of bacteria, moulds, yeasts, Lactic Acid Bacteria (LAB) and Enterobacteriaceae members at each stage of the "Otika" preparation were conducted by pour plating using Nutrient Agar (NA) for bacteria, Potato Dextrose Agar (PDA) for moulds, Malt Extract Agar (MEA) (supplemented with streptomycin sulphate) for yeasts, Man Rugosa Sharpe Agar (MRS) at pH 5.5 for LAB anaerobically and Eosin Methylene Blue (EMB) agar for the members of Enterobacteriaceae. Incubation was carried out with bacteria for 24 hr and LAB for 48 hr at 37°C and with fungi at 27°C for 48 hr. Colonies and spore forming units formed on the media were counted and subcultured. The Bacteria isolates were examined using microscopy, Gram staining, sugar fermentation test, biochemical tests, such as urease test, catalase test, citrate utilization test and indole test according to the methods of Fawole and Oso (2007) and Brenner *et al*. (2005) while fungal identification was done using the fungi conventional identification method. Each fungal isolate was microscopically examined by putting a drop of lactophenol-in-cotton blue on a clean glass slide. A sterile inoculating loop was used to transfer a small piece of the mycelium into the lactophenol. The mycelium was spread out carefully with the sterile needle, covered with cover slip and examined firstly with the low-power objective lens, then with the high-power objective lens of the light microscope for vegetative and reproductive bodies. The fungi were identified based on the morphologic characteristics of their mycelia and spores according to Deak and Beuchat (1994) and Sanni *et al*. (1994).

Test for Selected Positive and Negative Microbial Interactions between the Isolates

Mutualism/commensalism and antagonism were the respective positive and negative interactions determined between the microbial isolates. Agar Well Assay method with slight modification was employed to determine the exhibition of the mutualistic or commensalistic and antagonistic associations among the isolated microbes during the production of the "Otika". Muller Hillton Agar (MHA) was prepared and poured in Petri dishes. Cultures of microorganism were swabbed uniformly on the individual plates using sterile cotton swab. Well was bored using a sterile cork borer of 5 mm diameter and with micropipette, 1 mL of each test isolate was transferred into each well and incubated for 24 hours at 37°C (Benkerroum et al., 2004). The agar was examined for zones of inhibition which were measured in millimetres. Creation of inhibitory zone indicated antagonism and absence of zone of inhibition signified mutualism or commensalism. Every laboratory experiment was carried out aseptically.

Analysis of Data

Experiment was carried out in triplicate. Numerical data obtained were subjected to Analysis of Variance (ANOVA) and means were separated with Duncan's New Multiple Range Test at 95% confidence level using SPSS 16.0 version.

3. Results

Types, Occurrence and Population of Microbial Isolates during "Otika" Production

Based on the cultural, microscopic and biochemical characteristics, twelve and nine different species of bacteria and fungi were isolated during the preparation of "Otika," respectively (Table 1). Among the bacteria, two were *Bacillus* species; six were species of Lactic Acid Bacteria (LAB), three species belong to the family of Enterobacteriaceae and one species was in the genus *Staphylococcus*. Among the fungal isolates, one and three species belong to *Penicillum* and *Aspergillus*, respectively. Two species of yeasts were separately identified as *Saccharomyces* and *Candida*.

All bacteria and fungi except Enterobacter cloacae, Lactobacillus brevis and Leuconostoc mesenteroides were isolated on the sorghum grains. Bacillus subtilis, B. cereus, Staphylococcus aureus, Escherichia coli, Lactobacillus plantarum, Lactobacillus fermentum, Pediococcus acidilactici, Aspergillus flavus and all the yeasts were present throughout the steeping, malting and milling and at 24^{th} hour of fermentation, but Aspergillus flavus was absent at 24^{th} hour of fermentation (Table 1).

More of the isolated microorganisms disappeared towards the end (120 hours) of the fermentation. Reoccurrence of these microorganisms was observed for only *Bacillus subtilis* and *E. faecalis* at 96 hours and 120 hours of fermentation, respectively. *Lactobacillus plantarum*, *Saccharomyces cerevisiae* and *Candida tropicalis* showed 100% occurrence during the "Otika" production while isolates with the lowest level of occurrence (30%) were *Enterobacter cloacae*, *A. fumigatus* and *P. italicum*.

Table 1. The types and occurrence of bacteria and fungi isolated during the production of "Otika"

Microorganisms	Sorghum Grains	Steeping	Malting	Milling Boiling	Fermentation (Hr)					Number of time microbe Occurred (%)	
					24	48	72	96	120		
Bacillus subtilis	+	+	+	+	+	-	-	+	+	70	
Bacillus cereus	+	+	+	+	+	-	-	-	-	50	
Enterococcus spp	+	+	+	-	-	-	-	-	+	40	
Staphylococcus aureus	+	+	+	+	+	-	-	-	-	50	
Escherichia coli	+	+	+	+	+	+	-	-	-	60	
Enterobacter clocae	-	+	-	+	+	-	-	-	-	30	
Listeria monocytogenes	+	+	-	+	+	-	-	-	-	40	
Klebsiella spp	+	+	+	+	-	-	-	-	-	40	
Lactobacillus plantarum	+	+	+	+	+	+	+	+	+	100	
Lactobacillus fermentum	+	+	+	+	+	+	+	+	-	80	
Lactobacillus brevis	-	+	-	+	+	+	+	-	-	50	
Leuconostoc mesenteroides	-	+	+	+	+	+	+	-	-	60	
Pediococcus acidilactici	+	+	+	+	+	+	-	-	-	60	
Aspergillus flavus	+	+	+	+	-	-	-	-	-	40	
Aspergillus niger	+	+	+	-	+	+	-	-	-	50	
Aspergillus fumigatus	+	+	+	-	-	-	-	-	-	30	
Penicillium italicum	+	+	+	-	-	-	-	-	-	30	
Sacharomyces cerevisiae	+	+	+	+	+	+	+	+	+	100	
Saccharomyces species	+	+	+	+	+	+	+	-	-	70	
Candida krusei	+	+	+	+	+	+	+	-	-	70	
Candida tropicalis	+	+	+	+	+	+	+	+	+	100	

Legend: + = Present, - = Absent

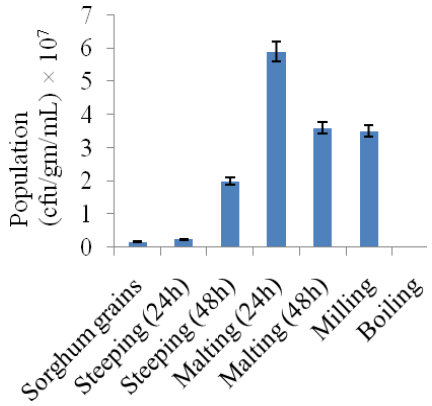

Figure 1a. Pre-fermentation total bacterial load

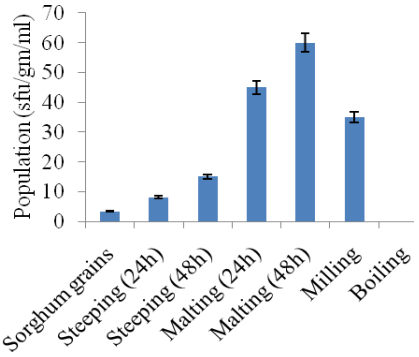

Figure 1b. Total bacterial load during fermentation of malted sorghum grains.

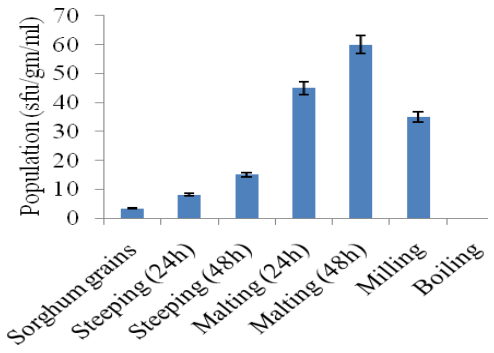

Figure 2a. Pre-fermentation fungal load

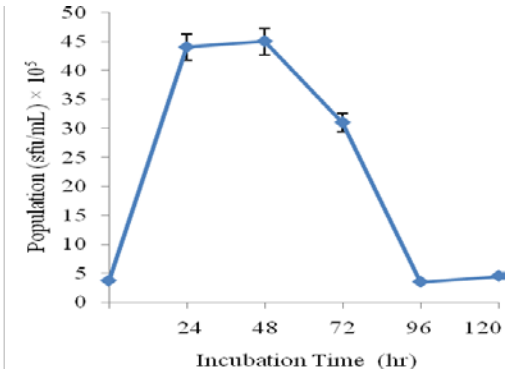

Figure 2b. Fungal load during the fermentationof malted sorghum grains

The total viable bacterial count increased significantly from 1.6 x 10^6 ± 0.33 cfu/gm/ml on the sorghum grains sample to 5.9 × 10^7 ± 0.05 cfu/ml during malting and later decreased to 2.5×10^7± 0.03 cfu/gm/ml after boiling. During fermentation, the population increased to 4.6 ×10^7 ± 0.33 cfu/gm/ml at 24 hr and decreased afterwards to 2.4 × 10^6 ± 0.05 cfu/gm/ml at 120 hr of fermentation (Figures 1a and b). Fungal population increased from 3.4 × 10^5 ± 0.05 cfu/gm/ml on the sorghum grains to 6.0 × 10^6 ± 0.05 cfu/ml after milling and declined to 0.0 sfu/ml during boiling (Figures 2a and b). During fermentation, the fungal load increased sharply after 0 hr at 3.7 ×10^5 ±0.03 cfu/ml to 48 hr at 4.5 ×10^6 ± 0.11 cfu/ml but dropped to 3.5 ×10^5 ± 0.11 cfu/ml at 96 hr of fermentation and again increased slightly (4.5 × 10^5 ± 0.11 cfu/ml) at 120 hr. Similar trend of change was recorded for the total viable lactic acid bacteria (LAB) population (Figures 3a and 3b), which increased significantly (<0.05) from 3.0 × 10^6 ± 0.0 cfu/gm/ml on sorghum grains to 3.2 × 10^7 ± 0.08 cfu/ml at 24 hr of malting. Then after, there was a reduction in the LAB population to 0 cfu/ml during boiling which increased to 9.0 × 10^7 ± 0.05 cfu/ml at 48 hr of fermentation and gradually decreased to 2.3 × 10^6± 0.0 cfu/ml at the end (120 h) of the fermentation. The total viable enterobacteriaceae load of the sorghum grains increased significantly from 1.5 × 10^6 ± 0.15 cfu/ml to 2.3 × 10^7 ± 0.08 cfu/ml during steeping (Fig. 4a). During the period of fermentation, there was an increase in this population to 4.2 × 10^6 ± cfu/ml at 0 hr which decreased to 3.7 × 10^7 ± 0.2 cfu/ml at 24 hr of fermentation (Fig. 4b). Then after, it decreased gradually and at 120 hr of fermentation the bacteria were undetectable.

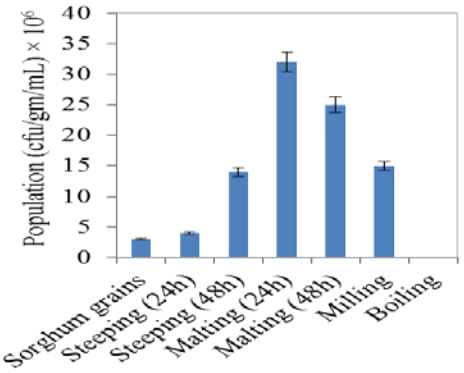

Figure 3a. Pre-fermentation lactic acid bacteria load

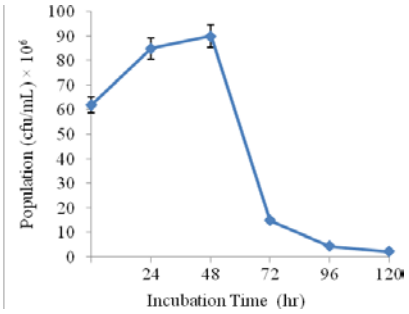

Figure 3b. Lactic acid bacteria load during the fermentation of malted sorghum grains

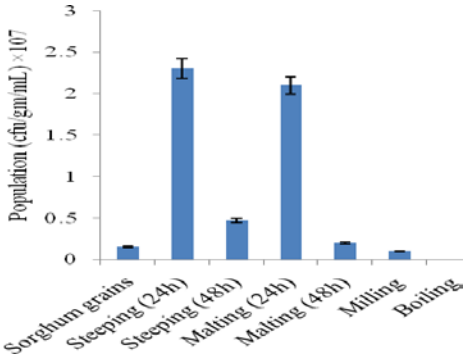

Figure 4a. Pre-fermentation enterobacteriaceae load

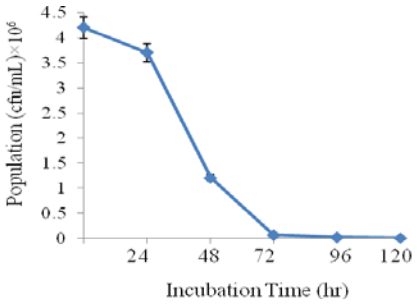

Figure 4b. .Enterobacteriaceae load during the fermentation of malted sorghum grains

Mutualism/Commensalism and Antagonism between the Lactic Acid Bacteria, Yeasts and Other Bacteria Isolated

Table 2 shows the interactions which existed between the bacteria, yeasts and Lactic Acid Bacteria (LAB). Lactic acid bacteria inhibited the growth of other bacteria but not its own members. The interaction between the yeasts and the LAB was positive as zone of inhibition was not created when they were co-cultured. Yeasts also showed a negative relationship against non-LAB bacteria by showing the zone of inhibition.

Table 2. Interaction between Lactic acid bacteria, yeast isolates and some of the bacteria isolated during "Otika" production

Microorganisms (LAB/Yeasts)	*Escherichia coli*	*Pediococcus acidilactici*	*Listeria monocytogenes*	*Lactobacillus fermentum*	*Bacillus subtilis*	*Staphylococcus aureus*	*Lactobacillus plantarum*	*Enterbacter faecalis*	*Enterococcus cloacae*	*Leuconostoc mesenteroides*	*Bacillus cereus*
Leuconostoc mesenteroides	+	-	+	-	+	+	-	+	+	-	+
Lactobacillus Fermentum	+	-	+	-	+	+	-	+	+	-	+
Lactobacillus Plantarum	+	-	+	-	+	+	-	+	+	-	+
Pediococcus acidilactici	+	-	+	-	+	+	-	+	+	-	+
Saccharomyces Cerevisiae	+	-	+	-	+	+	-	+	+	-	+
Candida tropicalis	+	-	+	-	+	+	-	+	+	-	+

Legend: LAB= Lactic Acid Bacteria

\+ = Zone of inhibition formed (Antagonism)

\- = Lack of zone of inhibition (Mutualism/Commensalism)

4. Discussion

Various species of microorganisms were isolated during the production of "Otika" which revealed the arrays of microorganisms. They exhibited cultural, cellular and biochemical properties similar to those described by Deak and Beuchat (1994) and Sanni *et al*. (1994) and Bergey's Manual of Systemic Bacteriology (Brenner *et al*., 2005); hence their probable names were as presented in Table 1. The presence of *Bacillus subtilis* at the beginning of fermentation of the milled malted grains suggests that the microorganisms were not completely killed during boiling; some could have also re-grown through their spores. *Bacillus subtilis* and *B. cereus* are spore formers and these spores help them to be resistant against heat that is, they can survive extreme temperatures. This was also confirmed by Boboye (2007) who worked on bacterial changes in sorghum larger beer where *Bacillus* spp. was isolated at the beginning of fermentation. *Lactobacillus plantarum* and two of the yeasts (*Saccharomyces cerevisiae* and *Candida tropicalis*) isolated were found throughout the production stages of "Otika". These *Lactobacillus* species and yeasts were reported to be predominant during cereal fermentation (Avicor *et al*., 2015; Kadjogbé *et al*., 2015). These data therefore mean that *L.* spp. and the yeasts play considerable roles in the fermentation of malted sorghum grains for "Otika" production. These roles might be similar to those played during cereal fermentation. The disappearance of some microorganisms during the production of the "Otika" may be attributed to the increased acidity and the lowered pH of the fermenting malted grains. Acid and lowered pH below 4 or 3 restrict the growth and survival of spoilage organisms and some pathogenic microorganisms, such as *Shigella*, *Salmonella* and *Escherichia coli* (Muyanja *et al*., 2003; Chelule *et al*. 2010; Nyanzi and Jooste, 2012; Rina and Sonali, 2016).

The increase in bacterial load at the initial stages before fermentation was a result of their dominancy due to favourable conditions. The studies carried out by some scientists showed these microorganisms are dominant fermenting microorganisms in fermented foods (Akinleye *et al*., 2014).

The increase in microbial loads before boiling indicates that the raw, steeped and malted grains contained appropriate nutrients for the microbes to utilize and multiply. This favourable condition could have resulted by the dominancy of bacteria at the initial stages before fermentation. Similarly, the fermenting malted, milled sorghum grains must have provided the microorganisms with sufficient and appropriate nutrients that caused the increase in their populations. Générose *et al*. (2016) who worked on "gowe", a fermented sorghum beer, reported that the volatile compounds observed during the primary fermentation stage supported an increase in the lactic acid bacteria and yeast counts. These compounds include alcohols, aldehydes, esters, hydrocarbon, furan, phenol, piperidine and acids; some of which were identified from 0 h of fermentation and may have been initiated during the stages preceding fermentation which are steeping and malting of sorghum kernels.

The later decrease in the loads of other bacteria besides LAB, after 24 hr of fermentation could be due to increased population of the LAB that must have produced acid causing reduction in pH (acidity) which seems to be detrimental to the mesophilic bacteria. A similar result has been reported by Babatunde and Oladejo (2014) and Teshome (2015) who posited that LAB produce many organic acids, such as lactic, acetic and propionic acids produced during fermentation as end products which provide an acidic environment unfavourable for the growth of many pathogenic and spoilage microorganisms. Acidic medium favoured yeast growth which underlies yeasts multiplication observed in the present study.

The mutual association that existed between yeasts and lactic acid bacteria has been noted in several cereal foods (Omemu *et al*., 2007; Omemu, 2011; Ogunbanwo *et al*., 2013). Enterobacteriaceae members are common on fermenting plant materials and have also been found in the natural fermentation of cereal products; thus their high load obtained before fermentation in the present work could be due to their possible presence on the sorghum grains from the farm where they were harvested. The bacterial isolates might have originated from the plants, utensils and vessels used previously during handling, malting, milling and fermentation. These data are similar to the report of Ogunbanwo *et al*. (2003). The presence of the microbes except *Aspergillus flavus* on the sorghum grains and during the fermentation implies that they are important for the production of "Otika". The differences observed in the level of occurrence of the fungi and bacteria mean that these microorganisms performed different

functions as they were associated with various stages of the "Otika" production. The presence of moulds at the initial stage of fermentation of cereal for "Ogi" production and the subsequent elimination was also reported previously Omemu *et al*. (2007). Fungi play vital roles at initial phase of fermentation mostly in saccharification of the substrates (Thapa and Tamang, 2004; Adebayo *et al*., 2014).

All LAB and yeasts evaluated in the present study showed antagonistic interaction to other isolated bacteria. This means that these microbes might have produced inhibitory metabolites. This conforms to the report of De Martinis *et al.* (2001) and Ogunbanwo *et al.* (2003) who reported that the antimicrobial compounds produced by LAB enable them to exert strong antagonistic activity against food contaminating microorganisms. None of the LAB isolates inhibited any of the yeast cultures. The relationship between yeasts and the entire LAB could therefore be mutualism or commensalism. Several authors have reported similar coexistence and positive interactions between yeasts and lactic acid bacteria in different African fermented foods (Omemu *et al*., 2007; Gulitz *et al*., 2013). The positive interaction between yeasts and LAB could have caused co-metabolism between them. Thus, the two groups of microbes appeared to have adapted to the food systems in the sorghum particularly the non-fermentable such as starch. This enabled them to adequately utilize substances in the fermenting sorghum thereby resulting to increased populations as observed in the figures. The stimulatory effect of yeasts on lactic acid bacteria during fermentation has been attributed to the provision of some compounds such as soluble nitrogenous compounds, B-vitamins, CO_2, pyruvate, propionate, acetate and succinate (Stadie *et al*., 2013).

5. Conclusion

The present study has provided useful information on the types of microbial communities and their associations that characterized the production of "Otika". *Lactobacillus plantarum*, *Saccharomyces cerevisiae* and *Candida tropicalis* were found to be predominant in the production of "Otika" and they have the ability to inhibit pathogenic microorganisms. There was re-occurrence of spoilage microorganisms after 72 hr which mean it start deteriorating after 72 hr which will not be safe for consumers. The present study also established the antimicrobial interaction between microorganisms which is either positive or negative. The information from this work would assist in the production of consistent quality of "Otika" beverage.

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No conflict of interest

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Isolation and Molecular Characterization of a Newly Isolated Strain of *Bacillus* sp. HMB8, With a Distinct Antagonistic Potential Against *Listeria monocytogenes* and Some Other Food Spoilage Pathogens

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Abstract

Antagonistic *Bacillus* sp. strain HMB8 and its concomitant *Micrococcus* sp. strain HMB7 were isolated from a healthy oral cavity of a volunteer. Phenotypic characteristics and 16S rRNA gene sequencing similarity confirmed that strain HMB8 belongs to *Bacillus* genus, whereas strain HMB7 belongs to *Micrococcus* genus. Isolated *Bacillus* sp. HMB8 exhibited considerable *in vitro* antagonistic activity against a broad range of indicator strains, including food spoilage bacteria, such as *Listeria monocytogenes*, *Staphylococcus aureus*, *Salmonella enterica Salmonella enterica* serovar Typhimurium, *Pseudomonas aeruginosa*, and *Escherichia coli*. Partial characterization of the bioactive compounds from the culture supernatant of the *Bacillus* sp. HMB8 was carried out using three extraction methods; ammonium sulfate, chloroform-methanol, and ethyl-acetate. All extracts retained good antibacterial activity against all indicator strains, but to varying levels. Overall, the ethyl-acetate extract of *Bacillus* sp. HMB8 demonstrated the highest inhibitory activity against most indicator strains, notably against *L. monocytogenes*. The findings of the present study suggest that *Bacillus* sp. HMB8 bioactive compounds may have potential biotechnological applications as food biopreservatives.

Keywords: *Listeria monocytogenes*, food-borne diseases, antagonistic activity, *Bacillus* spp., human oral bacteria, biopreservative.

1.Introduction

Food-borne diseases due to the consumption of contaminated and unsafe food products are among the leading causes of mortality worldwide, accounting for 20 million cases annually (Pal *et al*., 2016). In a comprehensive study, carried in 2012, food-borne illness was estimated to affect approximately 9.4 million people in the United States, leading to 1351 deaths each year (Scallan *et al*., 2011). Food poisoning occurs directly by bacterial food-borne pathogens, such as *Salmonella* spp., *Listeria monocytogenes*, *Escherichia coli*, and *Staphylococcus aureus*, or indirectly by their toxins (Kadariya *et al*., 2014; Saraoui *et al*., 2016).

Listeria monocytogenes is an opportunistic pathogenic psychrotrophic bacterium with public health significance. It is the etiologic agent of listeriosis, a severe food-borne illness with high rates of hospitalization and mortality (20% to 30%) (Saraoui *et al*., 2016; Seman *et al*., 2002). The majority (99%) of listeriosis cases have a food-borne source (Ivanek *et al*., 2005). Listerial contamination associated health problems have a significant economic impact, for example, listeriosis can impact Ready-To-Eat (RTE) foods costing more than a billion dollars a year (Ünlü *et al*., 2016). *L. monocytogenes* can tolerate and grow in many different unfavorable conditions, such as high salt concentrations, presence of CO_2, and freezing and storage at −18 °C. These features constitute challenges during conventional food preservation methods (Ivanek *et al*., 2005; Saraoui *et al*., 2016).

Bacillus genus is widely distributed in the environment and is of great significance in biotechnological and applied microbiology, especially for producing pharmaceuticals and chemicals for food production (Baruzzi *et al*., 2011; Borriss *et al*., 2011; Sumpavapol *et al*., 2010). Considerable research work has been oriented towards the isolation and the

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characterization of new novel bacteriocins and bacteriocin-like inhibitory substances from *Bacillus* genus. Bacteriocins offer several advantages that make them suitable for food biopreservation; they are effective against many food-borne pathogens, including *Listeria monocytogenes*, and become inactive when exposed to proteolytic enzymes in the gastrointestinal tract (Duarte *et al*., 2013; Sharma *et al*., 2011).

The human oral cavity and colon host the most diverse microbiome in the human body (Wade, 2013). The human oral cavity contains numerous distinct microbial niches, such as teeth, lips, tongue, cheek, hard and soft palates, and tonsils, which are densely populated by microbes, including viruses, fungi, protozoa, archaea, and bacteria (Dewhirst *et al*., 2010; Wade 2013). Based on traditional 16S ribosomal DNA investigation, approximately 690 different taxa of the human oral microbiome became publicly available on "The Human Oral Microbiome Database" website (www.homd.org). More than half of these taxa were officially named, while 32% are still considered as uncultivated phylotypes (Chen *et al*., 2010).

Recently, considerable attention has been given towards the isolation of new bacterial strains from different habitats, that may enable the discovery novel antimicrobial substances that can be used as natural food biopreservatives (Al-Dhabi *et al*., 2016; Axel *et al*., 2016; Crowley *et al*., 2013; Gómez-Sala *et al*., 2016; Manrique *et al*., 2016) and to combat multi-drug resistant pathogens (Asencio *et al*., 2014; Motta *et al*., 2004). The aim of the present study is the isolation and identification of *Bacillus* spp. from a healthy oral cavity of a volunteer, and the evaluation of the isolate's antagonistic potential towards *Listeria monocytogenes* and some other pathogenic species involved in food spoilage.

2.Materials and Methods

2.1. Indicator Strains

All the strains used as indicators belong to the collection of the Laboratory of Microbiology at Al-Hussein Bin Talal University, Jordan. All indicator strains were cultivated on Nutrient agar (Sigma-Aldrich) at 37 ºC for 18 hours, and stock cultures were maintained in Nutrient broth at –20 ºC with the addition of 40% glycerol. Indicator strains are listed in Table 1.

Table 1. Indicator strains used in this study

No	Indicator strains
1	*Listeria monocytogenes* (ATCC® 19115™)
2	*Staphylococcus aureus* (ATCC® 25923™)
3	*Salmonella enterica* subsp. *enterica* serovar Typhimurium (ATCC® 14028™)
4	*Pseudomonas aeruginosa* (ATCC® 10145™)
5	*Escherichia coli* (ATCC® 25922™)

2.2. Bacterial Isolation from Human Oral Cavity

Isolates were collected from the roof of the oral cavity of a healthy volunteer. Samples were obtained using sterile swab soaked with 0.85 % NaCl. The swab was streaked directly onto Nutrient Agar (NA) media (Sigma-Aldrich). After 24 hours of incubation at 37ºC, morphologically different colonies were isolated and sub-cultured by streaking on the surface of NA agar plate for purification and preservation.

2.3. Preliminary Antagonistic Activity Screening

Each isolated bacterial strain was preliminary screened for antagonistic activity towards all other concomitant isolated strains. Each isolated bacterium was individually grown in 15 mL in Mueller–Hinton (MH) broth (Oxoid) at 37 ºC for 16 h. Five microliters of each culture having and inoculum concentration of around 5×10^5 CFU ml^{-1} were spotted onto MH agar (Oxoid) plates. After 18 h at 37 ºC, the bacteria were killed by chloroform fumes, and the plates were sprayed with all other concomitant isolated bacterial cultures (100 µL of a previously grown culture in 15 mL of MH broth). The plates were incubated for a further 16 h at 37 ºC and any inhibition zones were reported.

2.4. Antagonistic Activity of Strain HMB8 towards Listeria Monocytogenes and Some Other Indicator Strains

Screening of inhibitory activity was studied by the agar spot test that involved growing of the bacterial strain HMB8 isolated from the human oral cavity in 15 mL of MH broth at 37 ºC for 16 h. Five microliters of HMB8 culture with around 5×10^5 CFU ml^{-1} inoculum concentration were spotted onto MH agar plates. After 18 h at 37 ºC, the bacteria were killed by chloroform fumes and the plates sprayed with the indicator strains cultures (Table 1) (100 µL of a previously grown culture in 15 mL of MH broth). The plates were incubated for a further 16 h at 37 ºC and the diameter of the inhibition zones was measured (in mm). Three replicate plates were used for each indicator strain.

2.5. Phenotypic Characterization of the Isolated Strains

Only strains that demonstrated an antagonistic potential were morphologically and biochemically characterized using standard techniques (Gram stain, colony shape, size, and color on NA plates, catalase, and oxidase test, etc.) according to Bergey's Manual of Determinative Bacteriology (Holt *et al.*, 1994). Growth characteristics at different temperatures and pH values were also studied.

2.6. Antibiotic Resistance Profiling

Isolates were subjected to antibiotic resistance profiling using the disc diffusion method on MH agar according to the CLSI procedures (Wayne, 2009). The following antibiotics (Oxoid) were used: neomycin 30 µg, chloramphenicol 30 µg, penicillin 10 µg, erythromycin 15 µg, tobramycin 10 µg, streptomycin 10 µg, kanamycin 30 µg, ampicillin 10 µg and gentamycin 10 µg.

2.7. Molecular Characterization of the Isolated Strains DNA Extraction

DNA extraction of isolated samples was performed using the G-spin Total DNA Extraction Mini Kit (iNtRON Biotechnology, Suwon, Korea) according to manufacturer's instructions. The extracted DNA was used as a template for PCR to amplify 16S rRNA genes.

2.8. Amplification of 16S rRNA Gene and Phylogenetic Analysis

The SSU rRNA gene was amplified with the bacterial forward primer 27F (3'- AGRGTTYGATYMTGGCTCAG-5') and reverse primer 1492R (5′-RGYTACCTTGTTACGACTT-3′). Amplification of 16S rRNA was performed in a total volume of 50 µL containing 2 µL Genomic DNA, 25 µL of 2x PCR Master-mix Solution (i-MAX II, iNtRON), 1 µL Forward Primer, 1 µL Reverse Primer, and 31 µL of sterile distilled water (sdH2O). PCR mixture with the addition of known bacteria genome, *Salmonella enterica* (ATCC® 14028™), was used as positive control, whereas PCR mixture with the addition of water in place of genomic DNA was used as negative control.

The PCR reaction mixtures, were incubated at 94°C for 3 minutes as an initial denaturation, cycled 30 times through denaturation for 1 minute at 94 °C, annealing for 1 minute at 60 °C, and elongation for 5 minutes at 72°C, and a final extension for 5 minutes at 72°C .Next, 10 µL of each PCR product was mixed with 2 µL of Blue/Orange 6x loading dye and analyzed via 1% agarose gel electrophoresis. In addition, 6 µL of 1 Kb Hyper ladder loading was used to identify band sizes. PCR quick-spin PCR Product Purification Kit (QIAGEN, Germany) was used to purify PCR products according to the manufacturer's protocol. The purified PCR products were sequenced using a commercial service provided by MACROGEN, Korea.

The resulting 16S rRNA gene sequences were compared with those in GenBank using the blast program (National Center for Biotechnology Information; NCBI) and the EzTaxon database (http://eztaxon-e.ezbiocloud.net/) (Kim *et al*., 2012). The 16S rRNA gene sequences of isolated strains were aligned with corresponding sequences of closely related type species (retrieved from the GenBank/EMBL/DDBJ database) using clustal x1.83 (Thompson *et al*., 1997).

Phylogenetic trees were constructed using the neighbor-joining (Saitou and Nei, 1987) contained in MEGA6 software (Tamura *et al*., 2013). Bootstrap values (more than 40%) based on 1000 replications were listed at nodes.

2.9. Nucleotide Sequence Accession Number

The nucleotide sequences of 16S rRNA documented in the present study were deposited in the NCBI nucleotide sequence database under Accession Numbers KX859179 (HMB7) and KX859180 (HMB8).

2.10. Partial Purification of Bioactive Compounds

To extract the bioactive compounds from the bacterial broth culture supernatant during the growth cycle, three methods of extraction were applied; ammonium sulfate extraction, chloroform-methanol extraction, and ethyl-acetate extraction. Strain HMB8 was inoculated into 1500 mL sterile nutrient broth and incubated on a shaker at 37°C for 72h. Cells were collected by centrifugation (4000 rpm for 15 min) and the supernatant recovered and passed through a 0.22 µm filter. The resultant supernatant was used in each of the extraction methods.

2.11. Ammonium Sulfate Extraction

Solid ammonium sulfate $(NH_4)_2SO_4$ was slowly added to 500 mL of the supernatant at 4°C to achieve a 70% saturated solution with constant stirring overnight. Precipitated bioactive compounds were pelleted by centrifugation (4000 rpm for 30 min, 4°C). The resultant pellet was re-suspended in 1/100 of the original culture volume in SP buffer (25 mM sodium phosphate buffer, pH 7). This solution was designated as a crude antibacterial preparation (ASE), and tested for its antimicrobial activity against indicator strains.

2.12. Ethyl-acetate Extraction

Five hundred milliliters of ethyl-acetate solvent were added to 500 mL of culture supernatant filtrate. The mixture was shaken vigorously for 15 min and kept stationary for another 15 min to separate the organic phase from the aqueous phase. The resulting mixture separated in two layers, the organic layer which contained the secondary metabolites and the aqueous layer. The crude extract (0.97 g) was obtained after concentrating the solvent using a rotary evaporator at 60 °C. This extract was designated as (EAE) and stored at 4 °C for the antimicrobial assay (Al-Dhabi *et al*., 2016). For determination of the extact's antimicrobial activity, it was re-suspended in dimethyl sulfoxide (DMSO).

2.13. Chloroform-Methanol Extraction

Five hundred milliliters of methanol were added to 500 mL of culture supernatant filtrate with vigorous stirring, then 125 mL of chloroform were added with vigorous stirring and incubated for 20 min on ice, and then the suspension was centrifuged at 14000 g for 5 mins. The upper aqueous layer was carefully removed, and the lower phase was dried. The crude extract was designated as (CME) and stored at 4 °C.

For determination of the extract's antimicrobial activity, it was re-suspended in SP buffer.

2.14. Extracts' Antibacterial Activity Determination

The antimicrobial activity of the resultant crude extracts was determined using the agar disk diffusion method (Kimura *et al*., 1998) against several indicator strains (Table 1), as well as against the concomitant isolated HMB7 strain. Dissolved extracts (ECE, CME and ASE) were sterilized by filtration through a 0.22 µm syringe filter (Millipore, Bedford, MA, USA). An aliquot (20 µL) of each dissolved extract was applied on discs (6 mm) and the discs were placed on MH agar plates previously inoculated with 100 µL of each of the ATCC indicator strains with around 5 × 105 CFU ml^{-1} inoculum concentration. After incubation at 37 °C for 16 h, MH plates were observed for the appearance of growth-inhibition zones around the discs.

3.Results

3.1.Isolation of Presumptive Antagonistic Bacteria from a Healthy Human Oral Cavity

Eight different bacterial colonies were isolated and sub-cultured on nutrient agar media on the basis of colony morphology. Next, each isolated strain was preliminary screened for an antagonistic activity towards all other concomitant isolated strains using the agar spot test. Only one strain designated as (HMB8) exhibited an antagonistic activity against another isolated strain designated as (HMB7) figure (1).

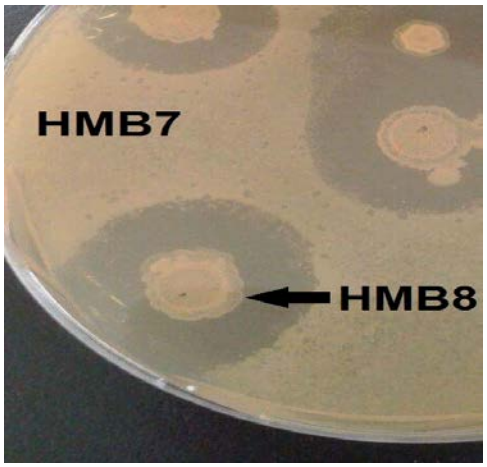

Figure 1. Screening of antagonistic activity of isolated bacteria as determined by the agar spot test. Bacteria designated (HMB8) showed an antagonistic activity towards one concomitant bacteria designated as (HMB7).

3.2.Antagonistic Activity of Strain HMB8 towards Listeria Monocytogenes and Other Indicator Strains

Strain HMB8 was tested for antagonistic activity against five ATCC bacterial food spoilage indicator strains, including *Listeria monocytogenes*, *Staphylococcus aureus*, *Salmonella* Typhimurium, *Pseudomonas aeruginosa*, and *Escherichia coli*. As shown in figure (2), HMB8 showed significant antagonism against all indictor strains except Salmonella Typhimurium; *E. coli* (26.1 ± 0.2 mm), *L. monocytogenes* (23 ± 0.9 mm), *S. aureus* (23 ± 0.9 mm), and *P. aeruginosa* (20.6 ± 0.7 mm).

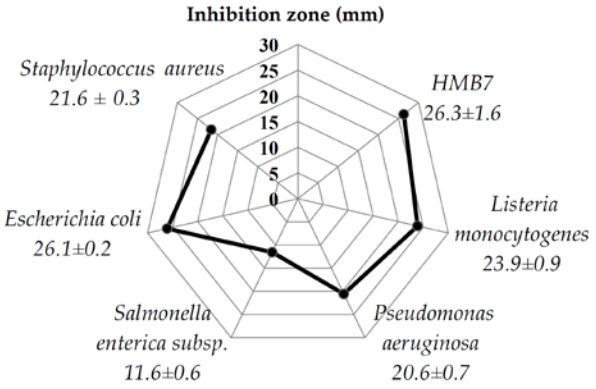

Figure 2. Inhibitory spectrum of HMB8 against *Listeria monocytogenes* and other indicator strains as determined by the agar spot test. The values are means of three replicates ± standard deviation.

3.3.Phenotypic Characterization of the Isolated Strains

On the basis of the results obtained during *in vitro* antagonistic activity screening for against food spoilage pathogens, both HMB8 and its concomitant inhibited HMB7 strains were morphologically and biochemically characterized using standard techniques. Growth parameters at different temperatures and pH values were also determined. The morphological and physiological characteristics of the selected strains are listed in Table 2.

Strain HMB8 was a Gram-positive bacillus. The colonies on agar media appeared white, circular, dry, flat, and irregular, with lobate margins. The optimum conditions for growth were 37°C and pH 8.0. In contrast, strain HMB7 was a Gram-positive coccus that produced yellow-pigmented circular colonies which were convex with entire margins. The optimum conditions for growth were 37°C and pH at 7.0. Biochemically, strain HMB8 was catalase and oxidase positive, had distinct fermentation profiles of different carbon sources, and was unable to produce urease. On the basis of the observed phenotypic characteristics, the strain was grouped into genus *Bacillus*. Strain HMB7 was catalase-positive, both oxidase and urease negative, and was able to use citrate; it was used as a sole carbon and energy source. Hence, the strain was grouped into genus *Micrococcus*.

Strain HMB8 was susceptible to neomycin, chloramphenicol, erythromycin, tobramycin, streptomycin, and gentamycin, and resistant penicillin and ampicillin. Strain HMB7 was susceptible to neomycin, streptomycin, kanamycin, ampicillin, gentamycin, erythromycin, and tobramycin, and resistant to both chloramphenicol and penicillin.

Table 2. Physiological and biochemical characteristics of strains HMB8 and HMB7.

Characteristics	HMB7	HMB8
Gram stain	Positive	Positive
Morphology	Coccus	Bacillus
Optimum growth pH	7.0	8.0
Optimum growth temp	37˚C	37˚C
Catalase production	Positive	Positive
Citrate utilization	Positive	Positive
Oxidase production	Negative	Positive
Urease production	Negative	Negative
Utilization of carbon sources		
D-Galactose	Negative	Positive
Sucrose	Positive	Positive
D-Fructose	Positive	Positive
D-Xylose	Negative	Positive
D-mannitol	Positive	Positive
Standard antibiotics (inhibition zone in mm)		
Neomycin 30 µg	19.3±0.2	30.9±1.3
Chloramphenicol 30 µg	Resistant	30.9±2.7
Penicillin 10 µg	Resistant	Resistant
Erythromycin 15 µg	9.3±1.3	23.9±1.9
Tobramycin 10 µg	14.9±0.2	31.1±0.7
Streptomycin 10 µg	33.5±0.6	37.8±1.1
Kanamycin 30 µg	32.8±0.6	41.4±2.2
Ampicillin 10 µg	24.1±2.8	Resistant
Gentamycin 10 µg	28.6±0.5	33.3±1.2

The antibiotics resistance profiles were determined using the disk diffusion assay. The values are means of three replicates ± standard deviation.

3.4. Molecular and Phylogenetic Analysis of the Isolated Strains

In assays for molecular identification of HMB7 and HMB8, PCR products of approximately 1.5 kbp were amplified from the 16S rRNA gene (Figure 3), and partial sequences of 961 bp and 1349 bp, respectively, were obtained (Genbank Accession Numbers: KX859179 (HMB7) and KX859180 (HMB8)). BLAST sequence comparisons suggested that HMB7 belongs to the genus *Micrococcus* and has a close relationship with *Micrococcus yunnanensis* YIM 65004$^{(T)}$, *Micrococcus aloeverae* AE-6$^{(T)}$, *Micrococcus endophyticus* YIM 56238$^{(T)}$ and *Micrococcus luteus* NCTC 2665$^{(T)}$. Nucleotide similarity was 99.27%, 99.17%, 98.96% and 98.96%, respectively. On the other hand, analyses of the 16S rRNA gene sequence of HMB8 indicated that it belongs to the genus *Bacillus* and was closely related to *Bacillus subtilis* subsp. *inaquosorum* KCTC 13429$^{(T)}$ and *Brevibacterium halotolerans* with 99.93% nucleotide similarity, each, followed by *Bacillus mojavensis* with 99.85% nucleotide similarity, and *Bacillus subtilis* subsp. *spizizenii* with 99.78% nucleotide similarity. The phylogenetic trees of both strains are illustrated in figures 4 and 5.

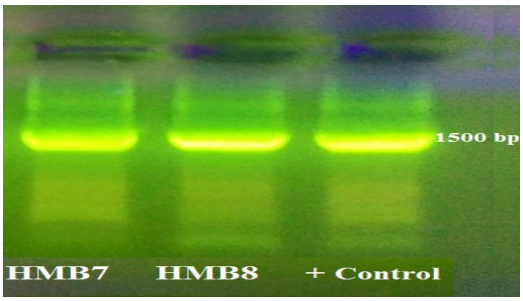

Figure 3. Agarose gel electrophoresis revealing successful amplification of 16S rRNA genes from HMB7 and HMB8.The 16S rRNA gene of *S.* Typhimurium was amplified as positive control.

In figure 4, the accession number of each type strain is shown in parenthesis. The tree was generated using the Neighbor-Joining (NJ) method using the MEGA6 software package and using *Nesterenkonia alba* DSM 19423 as an outgroup. Bootstrap values (more than 40%) based on 1000 replications are listed at nodes. Scale bar represents 0.02 substitutions per nucleotide position.

In figure 5, the accession number of each type strain is shown in parenthesis. The tree was generated using the Neighbor-Joining (NJ) method (Saitou and Nei 1987) using the MEGA6 software package (Tamura *et al*., 2013) and *Geobacillus thermoglucosidasius* NBRC 107763 as an outgroup. Bootstrap values (more than 40%) based on 1000 replications are listed at nodes. Scale bar represents 0.02 substitutions per nucleotide position.

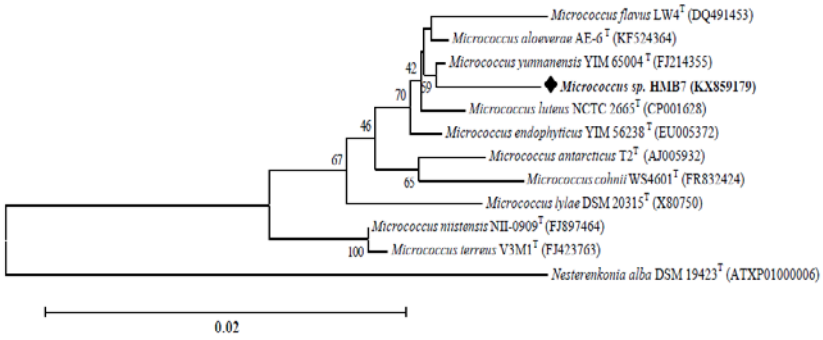

Figure 4. Neighbor-joining phylogenetic tree based on 16S rRNA gene sequences showing relationships between *Micrococcus* sp. HMB7 and the related type species of the genus *Micrococcus.*

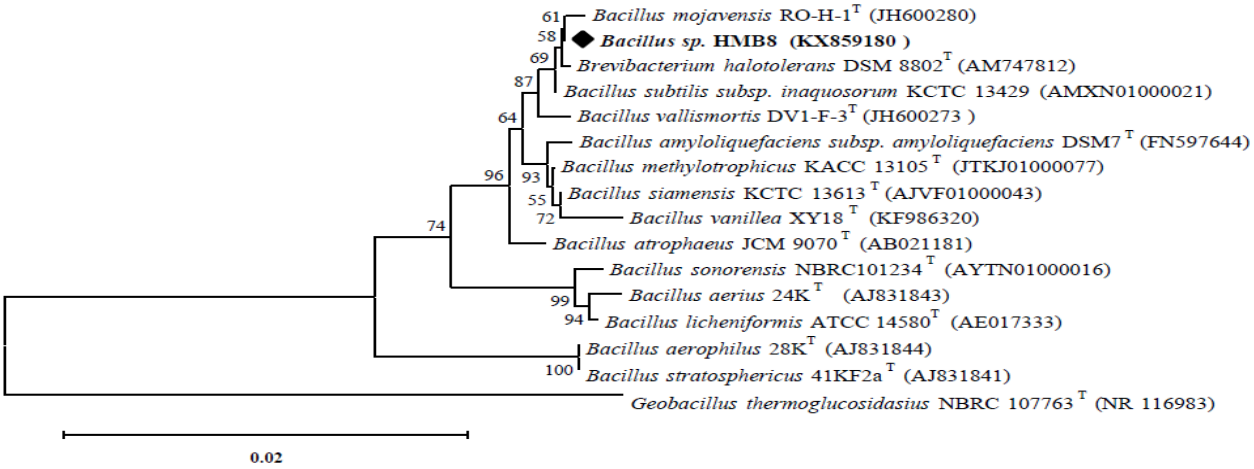

Figure 5. Neighbor-joining phylogenetic tree based on 16S rRNA gene sequences showing relationships between the strain *Bacillus* sp. HMB8 and the related type species of the genus *Bacillus*.

3.5. Bioactive Compounds Partial Purification and Antibacterial Activity

Table 3. Anti-bacterial activity of *Bacillus* sp. HMB8 semi-purified bioactive compounds.

Indictor strains	Strain number	Inhibition zone (mm)			
		Ethyl-acetate Extract (**EAE**)	Ammonium Sulfate Extract (**ASE**)	Chloroform-methanol Extract (**CME**)	Kanamycin 30 µg
L. monocytogenes	(ATCC® 19115™)	10.9±0.8	9.4±2.4	9.8±0.6	17.0
S. aureus	(ATCC® 25923™)	11.9±2.1	8.3±0.4	7.0±0.2	15.0
S. Typhimurium	(ATCC® 14028™)	8.0±4.1	7.2±1.0	6.8±0.3	15.0
E. coli	(ATCC® 25922™)	13.3±0.9	12.8±1.4	12.6±2.6	16.0
P. aeruginosa	(ATCC® 10145™)	9.3±0.2	11.4±0.6	9.1±0.2	7.0
Micrococcus sp. HMB7	Acc No (KX859179)	15.7±1.4	13.6±2.4	13.8±1.3	22.0

The values are means of three replicates ± standard deviation

After *Bacillus* sp. HMB8 strain was polyphasically characterized, an attempt was made to partially extract the bioactive compounds from its broth culture supernatant. Three methods of extraction were applied; ammonium sulfate extraction, chloroform-methanol extraction, and ethyl-acetate extraction. Resulting extracts were tested against all ATCC indicator strains (Table 1) as well as against the concomitant isolated *Micrococcus* sp. HMB7 using the agar disk diffusion method.

Results revealed also that the extracts retained a considerable antibacterial activity against all indicator strains and a good one towards the concomitant isolated *Micrococcus* sp. HMB7 (Table. 3).

4. Discussion

Bacillus spp. are widespread in natural environments (Porwal *et al.*, 2009) and have been and continue to be extensively screened for their potential production of useful bioactive products (Baruzzi *et al*., 2011; Borriss *et al*., 2011; Sumpavapol *et al*., 2010). In the present study, an attempt was conducted to isolate bacteria from a healthy human oral cavity with an antagonistic potential against *Listeria monocytogenes* and other food spoilage pathogens.

The human oral cavity is densely colonized by different bacterial species (Wade, 2013). It has been speculated that the population sizes of microorganisms in the human oral cavity are controlled by the antagonistic activity of the oral bacterial flora (Strahinic *et al*., 2007). Based on the above, we hypothesized that the human oral cavity is a source of bacterial strains with antagonistic activities, particularly towards food spoilage pathogens, as it is the gateway through which the food enters, and in which it gets chewed and mixed with saliva.

Strain HMB8 was isolated from the oral cavity and exhibited a noticeable antagonistic activity against another isolated strain designated as HMB). As shown in Figure (2), when HMB8 was subjected to further investigation against

five selected ATCC bacterial pathogens involved in food spoilage including *L. monocytogenes*, *S. aureus*, *S.* Typhimurium, *P. aeruginosa*, and *E. coli*, a wide spectrum of good antagonistic activities were observed.

Encouraged by these promising results, the two isolated strains were polyphasically characterized, the bioactive compounds were partially extracted from strain HMB8, and the antibacterial activities of the extracts were determined. On the basis of morphological, cultural, and some biochemical characteristics presented in table 2, strain HMB8 was initially identified as *Bacillus* spp., whereas HMB7 could be preliminary grouped into *Micrococcus* genus based on its morphology, and colony characteristics (creamy-yellow-color) (Kocur *et al*., 2006). Both strains were subjected to 16S rRNA gene amplification and sequencing. Molecular analysis results confirmed that HMB8 belongs to genus *Bacillus* and that it had the highest identity (99.93%) with *B. subtilis* subsp. *inaquosorum* and *Brevibacterium halotolerans*, followed by *Bacillus mojavensis* with 99.85% nucleotide similarity, and *Bacillus subtilis* subsp. *spizizenii* with 99.78% nucleotide similarity. All aforementioned species are considered members of the 'subtilis-group' or 'subtilis spectrum' (Rooney *et al*., 2009), which are difficult to differentiate as they possess similar phenotypic characteristics and share a high degree of 16S rRNA gene sequence similarity (Gatson *et al*., 2006; Roberts *et al*., 1996; Rooney *et al*., 2009). To reach a final accurate identification of strain HMB8, DNA–DNA hybridization and fatty acid composition of all closely related species should be evaluated. Unfortunately, such techniques are not available in our research lab. As clearly shown in figure 4, the detailed molecular phylogenetic analysis revealed that strain HMB7 represents a member of the genus *Micrococcus*. Although, high level of 16S rRNA gene sequence similarities is reported among *Micrococcus* spp. (Whitman, 2015), a combination of phenotypic and phylogenetic studies suggested that strain HMB7 is closely related to *Micrococcus yunnanensis* YIM 65004$^{(T)}$. Members of *Bacillus* and *Micrococcus* genera are common human oral cavity normal flora members and are frequently isolated from its different parts (Anesti *et al*., 2005; Bergan and Kocur, 1982; Chen *et al*., 2010).

An Initial attempt to purify the bioactive components from the HMB8 using three different extraction methods indicated that extracts retained a noticeable antibacterial activity against all indicator strains as well as against isolated *Micrococcus* sp. HMB7 (Table 3). The ethyl-acetate extract tended to produce the highest inhibitory effects against most used indicator strains, notably *L. monocytogenes* where a 10.9±0.8 mm zone of inhibition was observed. No obvious difference in the inhibitory effects of the extracts was found between Gram-positive and Gram-negative bacteria, suggesting that the bioactive compounds have a broad spectrum of activity.

One of the main characteristics shared among *Bacillus* spp. is the capacity to produce a large number of antimicrobial substances with different chemical compositions (Tabbene *et al*., 2009), *B. subtilis* subgroup is one of the key producers of these substances in the genus (Földes *et al*., 2000). A strain designated as *Bacillus subtilis* IFS-01 was isolated from the rhizosphere of cereals and showed antagonistic activity *in vitro* against various phytopathogenic, food-borne pathogenic, and spoilage micro-organisms (Földes *et al*., 2000). In another study, a new *Bacillus* strain producing a bacteriocin-like substance, with a broad inhibitory range for pathogenic and food spoilage bacteria, such as *L. monocytogenes*, was reported (Motta *et al*., 2007).

Due to the involvement in a number of food-borne diseases, *L. monocytogenes* constitutes a microorganism of high public health importance (Saraoui *et al*., 2016; Seman *et al*., 2002). The fairly broad inhibitory spectra of our of *Bacillus* sp. HMB8 strain against a number of serious and challenging food-borne pathogens/spoilage-causing bacteria, warrants further investigations to determine the possibility of using the extracted bioactive compounds as effective and safe biopreservative in food. Several attempts have been made to purify and characterize active compounds from *Bacillus* spp. and to evaluate their application in food biopreservatives (Baruzzi *et al*., 2011), for example, an extracellularly produced bacteriocin with desirable preservation attributes was isolated from food-grade *Bacillus subtilis* R75 (Sharma *et al*., 2011).

There have been several studies in the literature reporting antagonism among oral streptococci (Dempster and Tagg, 1982; Donoghue and Tyler, 1975; Strahinic *et al*., 2007). However, to our knowledge, no attempts were done to explore the potential antagonism among oral normal *Bacillus* spp. and to investigate their capacity to produce active substances that might be used in the food industry as biopreservatives.

The results obtained in the present study highlight the isolation oral *Bacillus* sp. HMB8 which demonstrated an ability to produce broad-spectrum inhibitory compounds that may prove useful as biopreservatives in different food products. Future studies should be conducted for the identification of the compounds found in the extract and to investigate their antagonistic activity (if any), to clarify their nature, and mode of action.

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