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Hilly MO, Adams MN and Nelson SC. 2009. Potential fly-ash utilization in agriculture. *Progress in Natural Sci.*, **19**: 1173-1186.

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Conferences and Meetings:

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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.

JJBS has been indexed by SCOPUS, CABI's Full-Text Repository, EBSCO, Zoological Records and National Library of Medicine's MEDLINE\ Pub Med system and others. I would like to reaffirm that the success of the journal depends on the quality of reviewing and, equally, the quality of the research papers published.

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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.

March, 2016

Prof. Ali Z. Elkarmi
Editor-in-Chief
The Hashemite University, Zarqa, Jordan

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Guest Editorial

Predatory Practices Are Increasing Among Some Open Access Medical and Biological Journals

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Sanjay A Pai discussed an important problem in the academic world and warns researchers about predatory publishers [1]. Devnani and his colleagues believe that low-income countries must devote more resources and more researcher support to aid in dealing with predatory journals [2]. According to these Authors, predatory journals are not indexed journals or, at least, they lack reputable indexes (such as "PubMed" [3]). These Journals declare, e.g.

We have contacted you earlier through email. Since we have not received any response from you regarding your valuable manuscript submission, we are taking the liberty of resending the invitation as we are aware that you may be engaged in other activities or my message may not have successfully reached you. I request you to submit a Research, Review, Case report or a Short commentary for December Issue. The impact factor of the journal is 1.9*.

What's that*? Nothing. It is written in the e-mail you have received, but if you go to their websites, you see that the declared IF is "an unofficial" IF. A fake Impact Factor, one of the stringent criteria to define a predatory journal by Beall Jeffrey [4]: it uses some made up measures, so claiming an exaggerated international standing.

We are now observing that predatory practices are also emerging among some reputable, indexed journals. Some Scopus, PubMed and Thomson Reuters indexed journals (especially journals indexed in Thomson Reuters' Zoological Index) seem to act like predatory journals, e.g., having unclear review processes and hidden publication fees. In effect, reviewing the last updates in Beall's list of predatory journals, we can find such journals [5]. It can undermine the credibility of research results and damage public trust in biological/medical journals.

In particular, we have observed questionable "special issues" in reputable indexed journals, as an example of these predatory practices [6]. It seems that some reputable indexed journals create special issues and publish many papers outside of the journal's scope apparently only to make money. Some open access journals which do not have a publication fee and have been supported by Universities or Institutes create these questionable "special issues" and publish many papers only to generate revenue. Figure 1 shows the number of published papers in questionable special issues by some reputable, indexed journals in the first half of 2015.

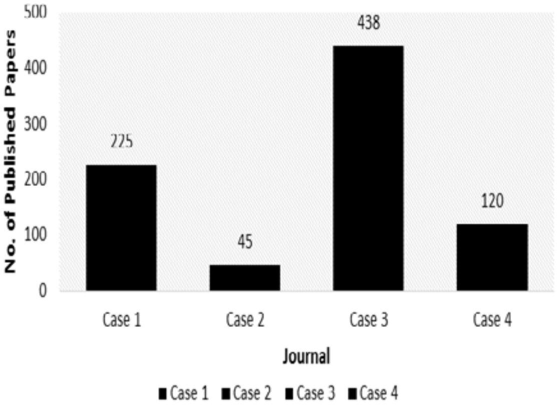

Figure 1. Number of published papers in questionable special issues (case: four different journals). Journals with predatory practice in the first half of 2015.

Here, we are defining "questionable special issues" as "issues with many papers in different domains" or "many special issues in the same year." Please note that predatory journals are improving their techniques of soliciting papers from authors. They send emails praising an author's earlier papers, inviting them to submit new papers. They enroll Editorial Board members to solicit papers from authors, or the Editorial Board members themselves are solicited to send papers every month.

To solve the problem of this predatory practice, researchers should be wary of open-access journals that organize many special issues or that frequently employ guest editors for themed issues.

In a less specific way, there are many potential unethical practices related to predatory journals, researchers need education and planning to confront them.

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Dadkhah M, Elias N, Jazi MD, Christova-Bagdassarian V and Abu-Elteen KH. 2015. A New Challenge in the Academic World: Earning Real Money and Eminence by Paper Publishing. Jordan Journal of Biological Sciences, **8**(2): 73 – 75.

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Estimation and Identification of Airborne Bacteria and Fungi in the Outdoor Atmosphere of Al-Mafraq Area, Jordan

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Abstract

Airborne bacteria and fungi were analyzed during November, 2013. Morbidity due to respiratory diseases was also reported. The studied zones include Al-Mafraq downtown, Al al-Bayt University, Al-Zaatari refugee camp and the open desert. A total of sixty air samples were collected by a microbiological air sampler on nutrient and tryptic soy agars as cultivation media for bacteria. Potato dextrose, Sabouraud dextrose and malt extract agars were used as cultivation media for fungi. Statistical analysis revealed that there was a significant difference between almost all studied zones ($P<0.05$). The highest bacterial level was detected in Al-Mafraq downtown with 2055 CFU m^{-3}, whereas the lowest level was detected in the open desert with 23 CFU m^{-3}. The highest level of fungi was detected in Al-Zaatari refugee camp (405 $CFUm^{-3}$), whereas the lowest level of fungi was observed in the open desert zone (13 $CFUm^{-3}$). Bacteria and fungi levels were within the suggested threshold value limits for culturable bacteria and fungi. Eleven different bacterial species and four fungal species were isolated from these zones and identified by biochemical and molecular techniques. Fungi were examined macroscopically and microscopically and compared to the morphology of published fungal species. The identified bacterial species were *Bacillus cereus*, *Bacillus aerius*, *Bacillus safensis*, *Bacillus subtilis*, *Bacillus axarquiensis*, *Bacillus pumilus*, *Bacillus amyloliquefaciens*, *Bacillus licheniformis*, *Bacillus methylotrophicus*, *Bhargavae acecembensis*, and *Cellulomonas* sp. The isolated bacteria were all aerobic, Gram-positive, endospore-forming bacteria and catalase positive. The identified fungi were *Aspergillus niger*, *Aspergillus fumigatus*, *Penicillium* sp. and *Fusarium* sp. In respect to respiratory diseases in the studied area, the most frequent lung diseases in the studied area was bronchitis (42%), followed by chest infection (25%), pneumonia (21%), and chronic obstructive pulmonary diseases (12%).In conclusion, the isolated microbial species may appear to originate from the dusts of human and animal.

Keywords: Airborne Bacteria, Air quality, Desert, Environment, Respiratory Diseases.

1. Introduction

It is well known that the outdoor air quality significantly affects the human health and ecosystem. At the global level, a sharp rise in outdoor air pollution was observed during the past decades (Ostro, 2004; Mandal and Brand, 2011; IARC, 2013). Outdoor air pollutants include various chemical compounds as well as several biological pollutants, especially airborne bacteria and fungi. It has been well documented that outdoor airborne bacteria and fungi as well as their spores are public health problem that affects the health of millions of people around the world (Ostro, 2004; Qudiesat *et al*., 2009; Menteşe *et al*., 2009; Mandal and Brand, 2011; Ko and Hui, 2012).

Several investigations have reported that soil, water, plants, animals and human are the main sources of outdoor air borne bacteria and fungi (Swan *et al*.,2002; Ostro, 2004; Menteşe *et al*., 2009; Abdul Hameed *et al*., 2009; Yassin and Almouqatea, 2010; Bowers *et al*., 2011; Hospodsky *et al*., 2012; Muhsin and Adlan, 2012; Ghosh *et al*., 2013).Based on these investigations, the diversity, distribution, and abundance of outdoor airborne bacteria and fungi were studied in several regions of the world and found to be diverse between any two cities, towns or villages around the world. These variations might be due differences in population size and density, and the type of activities within the examined areas. These mentioned variations can be very difficult to control.

Globally, the rising in the levels of outdoor airborne pathogens in highly populated areas is essentially a human ecological and health problem due to placing populations at risk of high burdens of respiratory diseases and other infectious diseases (Ostro, 2004; Qudiesat *et al*., 2009; Menteşe *et al*., 2009; Ko and Hui, 2012). For instance, outdoor airborne bacteria and fungi can cause several types of respiratory illnesses or conditions such as asthma, bronchitis, pneumonia, chronic obstructive pulmonary disease (COPD), seasonal allergies, and others (Yassin and Almouatea, 2010; Bowers *et al*., 2011). In addition, certain airborne bacteria and fungi or their endotoxins are known to induce infectious diseases, acute toxic effects, allergies and eye irritation in some individuals (Menteşe *et al*., 2009; Hospodsky *et al*., 2012). Outdoor airborne bacteria and fungi spores can

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easily travel through the air. Consequently, inhaling these microorganisms can impair human wellbeing and health and cause various respiratory diseases (Ostro, 2004; Qudiesat*et al*., 2009). Based on international reports, diseases associated with outdoor airborne bacteria and fungi can causes significant mortality and morbidity every year, particularly among children due to respiratory diseases (Ostro, 2004).

The causal links between the outdoor air quality and the levels of airborne bacteria and fungi, in any environment, are complex because they are often indirect, displaced in space and time, and dependent on a number of modifying factors, including the source types and the ambient physicochemical conditions (Ostro, 2004; Abdul Hameed *et al*., 2009; Dong and Yao, 2010; Bowers *et al*., 2011). More importantly, the survival and spreading of airborne bacteria and fungi is largely dependent on certain physicochemical factors like temperature and humidity (Lighthart and Stetzenbach, 1994; Brodie *et al*., 2007; Fierer*et al*., 2008). The airborne microorganisms usually thrive and circulate in damp and humid air conditions. Crowded conditions and poor air circulation can increase the spreading and the survival rate of these microorganisms and also increase the chance of people at risk of contagious respiratory diseases (Lighthart and Stetzenbach, 1994; Goyer *et al*., 2001; Ostro, 2004; Tsai and Macher, 2005; Brodie*et al*., 2007; Muhsin and Adlan,2012).

The levels of outdoor airborne bacteria and fungi were determined for different cities and countries around the world (Yassin and Almouqatea, 2010; Hospodsky *et al*., 2012; Muhsin and Adlan, 2012; Ghosh *et al*., 2013) and the threshold value limits for culturable bacteria and fungi were also suggested(Dong and Yao, 2010). The published data reveal that outdoor airborne bacteria and fungi were studied at the regional level (Yassin and Almouqatea, 2010; Muhsin and Adlan, 2012). However, at the local level, i.e., in Jordan, few studies were carried out to investigate the presence of the outdoor airborne bacteria and fungi. For instance, one study was conducted in northern Jordan (Saadoun *et al*., 2008) and another one was carried out in Zarqa city (Qudiesat*et al*., 2009) to analyze airborne microorganisms in hospitals.

The levels of outdoor airborne bacteria and fungi in Jordan are widely unexplored especially in the main cities, including Al-Mafraq governorate. This governorate is the second largest governorate in Jordan with respect to area but it is one of the lowest with respect to population (306,900 at the end of 2013) and population density (11.6 at the end of 2013) [DOS, 2015]. However, the population density and human activities are concentrated in certain areas in the governorate. It is also worth mentioning that the number of population is increasing due to the increase in the refugee's numbers after the Syrian crisis. For instance, the United Nations data indicated that the total persons of concern in Al-Zaatari refugeecamp are more than 84,000 at the beginning of 2015 (UNHCR, 2015). This number reflects an increase by about 30% in population size. Therefore, the present study was carried out to quantify and identify airborne bacteria and fungi in Al-Mafraq governorate in northern Jordan to establish standards for future reference. The levels of airborne bacteria and fungi were determined in the outdoor air of four selected zones in this governorate: Al-Mafraq downtown, Al al-Bayt University, Al-Zaatary refugee camp, and the open desert using different types of cultivation media. Furthermore, to examine if there is a link between inhalation or exposure to these airborne fungi and bacteria and development of respiratory symptoms or diseases, the morbidity rate, associated with respiratory diseases related to airborne bacteria and fungi,was also reported in the present study.

2. Materials and Methods

2.1. Description of Sampling Sites

In the present study, four different zones located in Al-Mafraq governorate were chosen for the collection of airborne bacteria and fungi. These zones were AL-Mafraq downtown (Zone A), Al al-Bayt University (Zone B), Al-Zaatari refugee camp (Zone C) and the open desert (Zone D) [Figure 1]. These selected sites differ basically in the population density, the type and the intensity of anthropogenic activities. Zone A is the commercial center of the city characterized by various types of human activities. Zone B, the only university in Al-Mafraq governorate, includes about 18500 persons. Zone C hosts more than 80,000 people (UNHCR, 2015). The last zone, Zone D is an open desert area, non-populated and has no human activities. Generally, Al-Mafraq governorate is semi-desert area in its nature. During sampling in November, 2013, the temperature was at daytime between 18-22°C and the humidity at that time was 34-38%.

2.2. Sampling and Cultivation of Airborne Bacteria and Fungi

A total of sixty air samples were collected in this study using microbial air sampler (M.A.Q.S.II-90, OXOID, UK). This device can hold 90 mm Petri dish containing media within an aluminum head of 380 holes. The air sampler device was set at an air sampling rate of 100 $\mathrm{Lmin^{-1}}$ per sample and at one meter tall. At the end of the sampling, the plates were removed and aerobically incubated at 37°C for 3 days in case of bacteria and at 25° C for 7 days in case of fungi. The aluminum head of the air sampler was sterilized by 70% alcohol between sample collections.

Five different types of microbiological media were used during the present study. Tryptic Soy Agar (TSA) and Nutrient Agar (NA) (HiMedia Laboratories Pvt. Mumbai, India) were used to cultivate airborne bacteria. Sabouraud dextrose agar (SDA), potato dextrose agar (PDA) and malt extract agar (MEA) (HiMedia Laboratories Pvt. Mumbai, India) were used to cultivate fungi and chloramphenicol was added to fungi media as a bacterial growth inhibitor.

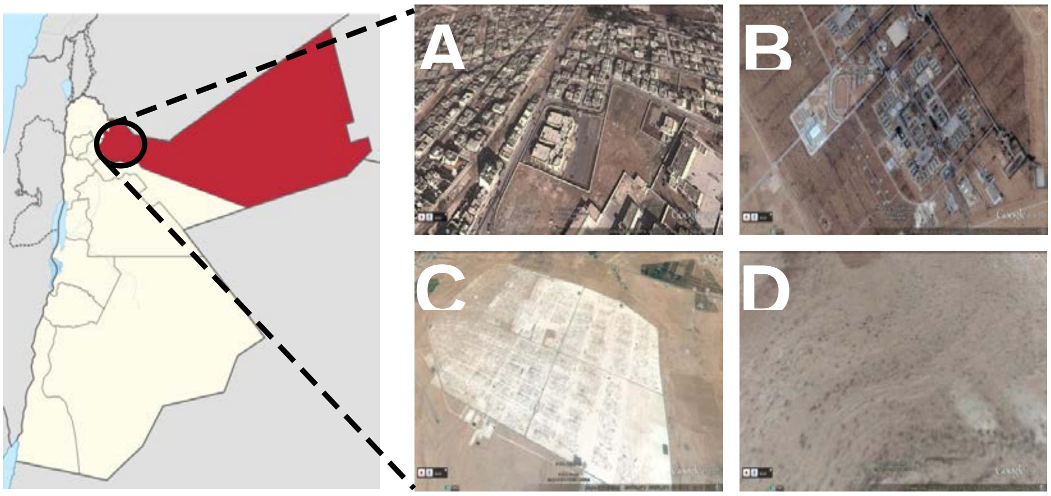

Figure 1. Map of the sampling sites: To the left, Jordan map with Al-Mafraq Governorate colored with red and a circle around the sampling area. To the right, aerial view of the sampling zones: (A) Al-Mafraq downtown (Zone A), (B) Al al-Bayt University (Zone B), (C) Al-Zaatari refugee camp (Zone C), and (D) the open desert (Zone D). Map and aerial views were retrieved from Google and Google Earth.

2.3. Enumeration and Isolation of Airborne Bacteria and Fungi

After the incubation of airborne bacteria and fungi as mentioned earlier, the developed colonies were counted and expressed as colony forming units per cubic meter (CFUm^{-3}).

The cultivated bacteria were compared with respect to colonial morphology, including shape, pigmentation, elevation, texture and other characteristics. Morphologically different colonies were transferred to new media and isolated as a pure culture. Additionally, 30% glycerol stock cultures were prepared from each isolate and stored at -20ºC (Jacob and Irshaid, 2012).The isolated fungi were subjected to macroscopic and microscopic examinations to observe their growth behavior, the nature of their mycelium and hyphae structure (Watanabe, 2002). Pure cultures of fungi were stored as tube slants at 4°C.

2.4. Identification of Airborne Bacteria

The bacterial isolates were first identified based on their reaction with Gram stain. Gram staining is essential to determine the further steps in identification to the species level. The bacterial isolates were then subjected to further identification using biochemical techniques.

First, the catalase test was conducted as a pre-requirement for the identification of bacteria by RapID CB® plus system. This test was performed by adding a few drops of the catalase test reagent (hydrogen peroxide) on a viable culture. The positive test leads to bubbles (oxygen) formation. This reaction can be seen with the naked eye.

Second, the RapID CB® Plus System (Remel, Lenexa, KS, USA) was used to identify the isolated strains to the species level. This system is mainly used for identification of Gram-positive bacteria. The system panel consists of four tests for utilization of carbohydrate and fourteen tests for single-substrate enzyme. Preparation of bacteria suspension of each isolate, inoculation, incubation times and temperatures, interpretation of reactions, and quality control were performed according to the manufacturer's recommendations for RapID CB® Plus system. Electronic RapID Compendium(ERIC) software was used to identify the isolated strains to the species level.

To identify those bacterial isolates that could not be identified by the biochemical method described above, the 16S rRNA gene sequencing was performed. In this method, DNA was extracted as previously mentioned (Jacob and Irshaid, 2012). Briefly, genomic DNA was extracted and purified from pure bacterial culture from each isolate using the EZ-10 Spin Column Genomic DNA (Biobasic, Ontario, Canada) following the instructions of the manufacturer.

Pure DNA samples were then subjected to 16S rRNA gene sequencing by GENEWIZ, Inc., USA.The16S rRNA gene sequences of the new isolates were deposited in GenBank® database. The resulting 16S rDNA sequences were analyzed to identify these strains by comparison with the complete nucleotide collections obtained from GenBank® database using Web BLAST Service (http://blast.ncbi.nlm.nih.gov /blast/Blast.cgi).

2.5. Fungal Identification

The colonial morphology of fungi was examined using a stereomicroscope (Meiji Techno Co., Ltd, Japan) and then wet mounts in lacto-phenol cotton blue were prepared for microscopic examination. The observations were then evaluated and compared to what is documented in the literature (Watanabe, 2002).

2.6. Statistics of Morbidity

The present study is also interested in investigating the relationship between outdoor airborne bacteria and fungi and respiratory illnesses or conditions especially as bronchitis, chest infection, pneumonia, asthma and COPD. Thus, data about respiratory diseases were collected from Al-Mafraq governmental hospital. The data represent all the admitted respiratory diseases cases in this hospital during2013.

2.7. Statistical Analysis

A statistical analysis was carried out using SPSS 19.0 software. Data of the bacterial counts underwent one-way ANOVA test and data of fungal counts underwent two-way ANOVA test. The means were compared using Duncan's multiple range tests at significance level of 5%. Microsoft Excel 2010 was used to calculate the standard deviation (SD) and standard error of the mean (SEM) and preparing the graphs.

3. Results

The present work was undertaken to quantify and identify the outdoor airborne bacteria and fungi in four different sites in Al-Mafraq governorate. The studied sites were Al-Mafraq downtown, Al al-Bayt University campus, Al-Zaatari refugee camp and the open desert. A total of sixty samples were collected and studied from these sites. The temperature values in the selected sites ranged from 18-22 °C during the sampling time, whereas the relative humidity was within the range of 34-38%.

3.1. Levels of Airborne Bacteria and Fungi

The quantitative analysis indicated that the level of bacteria in all the four studied zones range from 50 to 2055 CFUm^{-3} when NA was used as the cultivation medium. The highest bacterial level was detected in Al-Mafraq downtown zone, whereas the lowest level was detected in the open desert zone (Figure2). The statistical analysis indicated that there was a significant difference ($P<0.05$) in bacterial levels between all zones ($P<0.05$). However, when TSA was used as the cultivation medium, the levels of bacteria was found to range from 23 to 1263 CFU m^{-3}. The highest level of bacteria was detected in Al-Zaatari refugee camp, whereas the lowest level of bacteria was detected in the open desert, when TSA was utilized for growth (Figure 1). Statistical analysis indicated that there wasa significant difference ($P<0.05$) in the bacterial levels between the four studied zones when TSA was used as the cultivation medium, except between zone A and C ($P>0.05$).

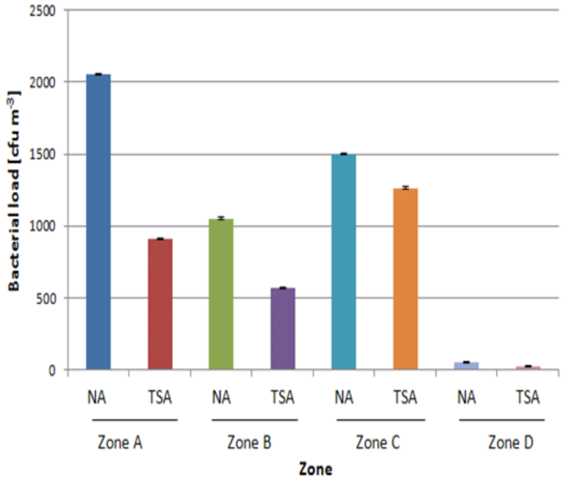

Figure 2. Bacterial levels (CFUm^{-3}) in outdoor air of the studied zones (A, B, C, and D) according to the type of medium used for their cultivation. CFU: colony forming unit; TSA: tryptic soy agar; NA: nutrient agar. Data represent the mean of three separate measurements.

In respect to the levels of airborne fungi, the statistical analysis indicated that there was a significant difference in the fungal levels between the four zones ($P<0.05$). The level of fungi ranged from 13 to405 CFU m^{-3}, when MEA was used as the cultivation medium. The highest level of fungi was detected in Al-Zaatari refugee camp zone, whereas the lowest level of fungi was observed in the open desert zone. When SDA was used for fungal growth, the level of fungi decreases and ranged from 14 to 295 CFUm^{-3}. The highest number of fungal count was found in Al-Mafraq downtown, whereas the lowest number of fungi was detected in the open desert. Moreover, when PDA was used as the cultivation medium, the level generally decreased and ranged from 16 to 191 CFU m^{-3}. The highest level of fungal colonies was detected in Al-Zaatari refugee camp zone, whereas, the lowest level of fungal colonies was found in the open desert zone (Figure 3).

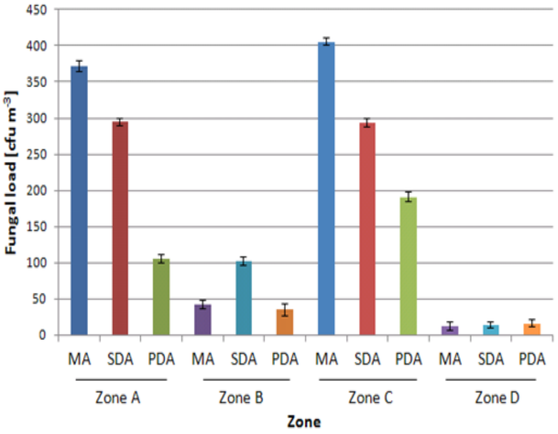

Figure 3. Fungal levels (CFU m^{-3}) in the outdoor air of the studied zones (A, B, C, and D) according to the type of media. CFU: colony forming unit; MEA: malt extract agar; PDA: potato dextrose agar, SDA: Sabouraud dextrose agar.

3.2. Identification of Airborne Bacteria and Fungi

A total of seventy two isolates of bacteria and fungi were isolated. The isolates were found to represent eleven different bacterial species and four fungal species. Eleven morphologically different bacterial isolates were selected from these examined zones. These eleven isolates were designated as TSA1.3A, TSA1.3C, TSA1.4C, TSA2.3D, NA3.1A, NA2.5B, NA2.4B, NA3.4C, NA3.5IC, NA3.2C and NA3.5IIC. The TSA or NA in the beginning of isolate code refers to TSA or NA medium used for their cultivations, whereas the letter at end of the isolate code (A, B, C or D) refers to zone from which the isolate was obtained. Six of these eleven isolates were recovered from Al-Zaatari refugee camp samples (zone C). Two isolates were recovered from each of Al-Mafraq downtown (zone A) and Al-al-Bayt University campus(zone B) samples. Only one isolate was obtained from sample collected from the open desert (zone D).

Morphologically, all the recovered isolates were shown to be Gram-positive aerobic bacteria. These isolates were also subjected to biochemical identification by RapID CB$^{®}$ plus system and ERIC software. The biochemical properties of the isolated bacterium are shown in Table 1. All the tested isolates gave a positive reaction with catalase test as well as positive for utilization of glucose and potassium nitrate. Furthermore, all examined isolates were able to hydrolyze p-nitrophenyl-β,D-glucoside, p-nitrophenyl-glycoside and the fatty acid ester. Based on the data generated from these biochemical analyses and using ERIC software, only one bacterial isolate (TSA1.3A), out of eleven, could be identified by this method. TSA1.3A isolate was identified as *Cellulomonas* sp. with 99% probability.

Table 1. Qualitative biochemical tests of the isolated strains and their identification results using ERIC software.

Test	Result
GLU*	+
SUC	+
RIB	-
MAL	-
αGLU	-
βGLU	+
NAG	-
GLY1	+
ONPG	-
PHS	-
EST	+
PRO	-
TRY	-
PYR	-
LGLY	-
LEY	-
NIT	+
CAT	+
PIG	-

***Abbreviations of chemical tests**: +: Positive reaction; -: Negative reaction. GLU: Utilization of Glucose, SUC: Utilization of Sucrose, RIB: Utilization of Ribose, MAL: Utilization of Maltose, αGLU: Hydrolysis of p-Nitrophenyl-α,D-glucoside, βGLU: Hydrolysis of p-Nitrophenyl-β,D-glucoside, NAG: Hydrolysis of p-Nitrophenyl-n-acetyl-β,D-glucosaminide, GLY1: Hydrolysis of p-Nitrophenyl-glycoside, ONPG: Hydrolysis of o-Nitrophenyl-β, D-galactoside, PHS: Hydrolysis of p-Nitrophenyl phosphate, EST: Hydrolysis of the fatty acid ester , PRO: Hydrolysis of Proline-β-naphthylamide, TRY: Hydrolysis of Tryptophan-β-naphthylamide, PYR: Hydrolysis of Pyrrolidine-β-naphthylamide, LGLY: Hydrolysis of Leucyl-glycine-β-naphthylamide, LEU: Hydrolysis of Leucine-β-naphthylamide, URE: Hydrolysis of Urea, NIT: Utilization of Potassium nitrate, CAT: Catalase test, and PIG: Yellow Pigmentation.

Biochemical tests were not enough to identify most of the isolated strains. Therefore, for the remaining unknown isolates, DNA was isolated and subjected to molecular analysis, namely, 16S rRNA gene sequencing and analysis. The unknown isolates TSA2.3D, TSA1.4C, NA3.1A, NA2.5B, NA2.4B, NA3.4C, NA3.5C, NA3.2C, TSA1.3C and NA3.5IIC have 97% identity or more with *Bacillus methylotrophicus*, *Bacillus axarquiensis*, *Bacillus cereus*, *Bhargavaea cecembensis*, *Bacillus safensis*, *Bacillus pumilus*, *Bacillus amyloliquefaciens*, *Bacillus licheniformis*, *Bacillus aerius* and *Bacillus subtilis*, respectively. All 16S rRNA sequences were deposited in GenBank® database. All isolates and their identifications as well as their GenBank database accession numbers are listed in Table 2.

Table 2. The closest relatives of the isolated airborne bacteria based on 16S rRNA gene sequence as well as the GenBank® accession number (GBAN) of the sequences of the isolated airborne bacterial species.

Isolate code	Identification	Identity (%)	GBAN
TSA2.3D	*Bacillus methylotrophicus*	97	KP297807
TSA1.4C	*Bacillus axarquiensis*	99	KP297808
NA3.1A	*Bacillus cereus*	97	KP297809
NA2.5B	*Bhargavaeacecembensis*	99	KP297810
NA2.4B	*Bacillus safensis*	98	KP297811
NA3.4C	*Bacillus pumilus*	98	KP297812
NA3.5C	*Bacillus amyloliquefaciens*	99	KP297813
NA3.2C	*Bacillus licheniformis*	99	KP297814
TSA1.3C	*Bacillus aerius*	97	KP297815
NA3.5IIC	*Bacillus subtilis*	98	KP297816

3.3. Identification of Airborne Fungi

Four morphologically different fungal isolates were isolated from the four selected zones using three different media (SDA, PDA and MEA). These fungal isolates were designed as SDA1.1D, PDA3.1A, PDA1.2C and MEA2.2A. Based on macroscopic and microscopic examinations, the SDA1.1D, PDA3.1A, PDA1.2C and MEA2.2A were classified as *Aspergillus niger*, *Aspergillus fumigatus*, *Penicillium* sp. and *Fusarium* sp., respectively. *Aspergillus fumigatus* and *Fusarium* sp. were isolated from the samples taken from Al-Mafraq downtown zone, whereas *Penicillium* sp. and *Fusarium* sp. were recovered from the samples collected from Al-Zaatari refugee camp zone and the open dessert zone, respectively.

3.4. Annual Morbidity Due to Respiratory Diseases in the Studied Area

The data collected from Al-Mafraq governmental hospital for the year 2013 indicated that the highest percentage of respiratory diseases was bronchitis (42%, 100 cases). This was followed by chest infection (25%, 60 cases), pneumonia (21%, 50 cases) and COPD (12%, 30 cases) [Figure 4].

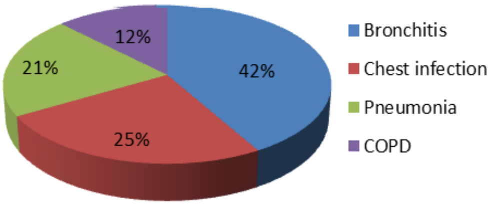

Figure 4. Annual rate of respiratory diseases among admissions to Al-Mafraq governmental hospital in the year 2013.

4. Discussion

In the present study, experiments were conducted to determine the levels of microorganisms (mainly bacteria and fungi) in four selected zones in Al-Mafraq governorate and characterize themto species level. In addition, the present study tends to examine the link between the presence of these airborne bacteria and fungi and development of respiratory diseases. Thus, the respiratory diseases in the same area were also reported.

The results of the present study indicated that there were significant differences in the number of bacteria and fungi among the tested zones. The levels of bacteria and fungi were found to be related to the population density as well as to the human activities and traffic in the studied zones. This conclusion is true when the crowded zones (Al-Mafraq downtown, Al al-Bayt University, and Al-Zaatari refugee camp) were compared to the zone of low or no human activity (the open desert). Human activities seem to be the main generator of outdoor bioaerosols as indicated by many reports (Menteşe *et al*., 2009; Ostro, 2004). Different human activities can contribute in generating or increasing the bioaerosol levels. These include shedding of skin cells, talking, coughing, and sneezing. Sneezing is one of the most vigorous mechanisms of spreading airborne microorganisms by generating as many as two millions of droplets per sneeze (Krishna, 2004). The presence of such droplets or particulates in air adds protection to bacterial cells and result in enhanced survival of the airborne microorganisms. Additionally, both humans and animals release small skin fragments from the body containing different bacterial species. Humans walking will generate up to 5,000 bacteria per minute to the surrounding air (Smith, 2006).

The highest level of bacteria was detected in the atmosphere of Al Mafraq downtown with 2055 CFUm^{-3} when NA was used as the cultivation medium and 1263 CFUm^{-3} when TSA was used. The threshold value limit for the culturable bacteria was suggested as 5000-10000 CFU m^{-3} (Dong and Yao, 2010). The levels of bacteria in all zones and the use of different media did not exceed this suggested threshold. However, caution should be taken when the results of different studies are compared due to differences in the geographic zone, season and time of sampling, media of cultivation, type and intensity of human activity, growth cycle of organisms, and meteorological factors (Abdel Hameed *et al*., 2009; Dong and Yao, 2010). For comparison, higher number of bacteria (12,639 CFU m^{-3}) was observed in the train stations and subway system in Beijing (Dong and Yao, 2010). The Beijing environment is characterized by being among the highest density and intensity of human activity compared to our selected region. In the open commercial streets of Beijing, the number was much lower than those reported for the train stations and subway system, supporting the role of human density and activities in the obtained results.

Furthermore, eleven bacterial species and four fungal species were isolated from the outdoor air environment of the four selected zone areas in this governorate. The isolated bacteria were found to belong to the following species: *Cellulomonas* sp., *Bacillus methylotrophicus*, *Bacillus axarquiensis*, *Bacillus cereus*, *Bhargavaea cecembensis*, *Bacillus safensis*, *Bacillus pumilus*, *Bacillus amyloliquefaciens*, *Bacillus licheniformis*, *Bacillus aerius* and *Bacillus subtilis*. The isolated bacteria are mostly typical airborne bacteria and possess characteristics that provide them with resistance to harsh environmental conditions and dispersal ability. These isolated bacterial species were all Gram-positive and endospore-forming. Gram-positive bacteria are in general more resistant to drying than Gram-negative bacteria because of their thick and rigid cell wall (Madigan *et al*., 2009). Furthermore, the endospores of endospore-forming bacteria are extremely resistant to drying and promote survival in air (Goyer*et al*., 2001). It is also clear that most of the isolated genera belong to the genus *Bacillus* which is a typical spore-forming bacterium. The spores of this genus are characterized by their resistance to dryness and UV radiation. These suitable characteristics will ultimately increase the chance of these species to grow and thrive on these examined areas.

It has been reported that Gram-positive bacteria seem to predominate in dusts of animal and human origin, whereas Gram-negative bacteria predominate in dusts of plant origin (Swan *et al*., 2002). Because all of our isolates were Gram-positive, this supports the aforementioned assumption that the detected species and levels of bacteria are mainly due to human presence and its activities and density. Similarly, certain studies in Europe have demonstrated that Gram-positive bacteria are the most commonly found bacteria in indoor air environment (Gorny and Dutkiewicz, 2002). In addition, other study on US office buildings revealed that Gram-positive cocci are the most prevalent in both indoor and outdoor air (Tsai and Macher, 2005).

In respect to the levels of airborne fungi, the statistical analysis indicated that there was a significant difference in fungal levels between the four zones ($P<0.05$). The levels of fungi ranged from 13 to 405 CFU m^{-3}, when malt extract agar was used as cultivation medium. When SDA was used as cultivation media, the levels of fungi decreased, ranging from 14 to 295 CFU m^{-3}. When PDA was used as cultivation media, the levels generally decreased and ranged from 16 to 191 CFU m^{-3}. The threshold value limit for the culturable fungi was suggested as 5000-10000 CFU m^{-3} (Dong and Yao, 2010). These data revealed that the reported level of fungi in the examined areas are generally far below the suggested threshold value limit. In other parts of the world, fungi level was found to be 1528 and 1806 in train stations and the subway system in Beijing respectively, whereas, in open commercial streets of Beijing, the calculated level was very low (Dong and Yao, 2010). Based on our data, the values of certain physicochemical factors are closely similar, including the temperature and humidity. Taken together, these findings indicated that the levels of fungi might be due to human presence and its activities and density.

The data generated from this study also revealed that the *Aspergillus niger*, *Aspergillus fumigatus*, *Penicillium* sp., and *Fusarium*sp. were only identified fungi species in atmosphere of these examined areas. *Aspergillus niger* and *Aspergillus fumigates* are usually associated with two respiratory diseases in humans, which are known as allergic and invasive aspergillosis. These species seem to be the most frequently isolated airborne species of fungi in other regions. For instance, the common genera of fungi frequently isolated from the air of hospitals at the United Arab Emirates were *Aspergillus* and *Penicillium* (Jaffal *et al*., 1997). Similarly, *Aspergillus* and *Penicillium* were also among the frequently detected species in the industrial town of Helwan, Egypt (Abdel Hameed *et al*., 2009). Recent study also revealed thatthe genera *Aspergillus*, *Penicillium,* and *Fuzarium*were isolated from the outdoor air of the Basrah city of Iraq (Muhsin and Adlan, 2012).Thus, these findings are in agreement with our findings. In addition, these findings and our findings suggest that the potential source of these fungi species is likely to be the similar. However, a closer inspection of the aforementioned areas revealed that some of the environmental factors among these areas are not necessarily alike or the same.

It is worth mentioning that the level and distribution of these airborne bacteria and fungi species among the tested zone areas were not uniform or similar; rather, each species appeared to be associated with a certain zone. This is in consistence with the previous studies which showed that the cell concentrations of airborne bacteria and fungi species can be affected by various environmental factors (Ostro, 2004; Menteşeet al., 2009; Yassin and Almouqatea, 2010; Mandal and Brand, 2011; Muhsin and Adlan, 2012). These factors include temperature, humidity, air dust, soil dirt, sanitary conditions as well as human presence, activities and density. Type of cultivation medium, sampling location and height from which these samples were collected can also influence the level and distribution of these airborne microorganisms. A closer look at these four examined locations revealed that there were high similarities regarding these factors, with exception human presence, density and type of activities as well as the sanitary conditions. Therefore, the observed variations in levels and distribution of these airborne species are more likely due to human density and activities as well as due to the sanitary conditions of the tested areas.

Based on the data generated from the present study, only a small number of outdoor airborne fungal and bacterial species were identified in our selected areas by using cultivation-dependent techniques. Therefore, it is possible to speculate that the exact number of the species of fungi and bacteria in our air samples are likely to be underestimated or overlooked by the cultivation-dependent methods. Hence, the full extents of the outdoor airborne bacterial and fungal diversities in the examined zones remain poorly characterized and understood.

Data about respiratory diseases were also collected exclusively from Al-Mafraq governmental hospital for the year 2013 during this study. The highest percentage of these diseases was bronchitis (42%, 100 cases). Bronchitis is the inflammation of bronchial tubes. The main causes of bronchitis are viral; however, airborne bacteria may cause bronchitis, especially in people underlying health problems (Warrel, 2008). Chest infection was the second most frequent respiratory disease (25%, 60 cases) that was recorded by Al-Mafraq governmental hospital. Chest infection is the infection of lungs or airways and it has two main types: bronchitis and pneumonia. Pneumonia represents 21% of respiratory diseases. It is an inflammation of lungs that is usually caused by an infection. One type of pneumonia occurs when aerosols are inhaled into lungs (called aspiration pneumonia). Bacteria are among the common cause of pneumonia in adults (Meterskye*t al*., 2012). However, the detected species are not among the main causes of pneumonia. A COPD is the least frequent respiratory disease. Causes of COPD are almost smoking and/or air pollution. A previous study also reported that outdoor air pollution was associated with the development of COPD (Ko and Hui, 2012). Taken together, it appears that the isolated species do not correspond directly to the reported cases of respiratory diseases in this governorate during the year 2013. Nonetheless, the resident of the examined areas may face some health problems due to the continuous inhalation of or the exposure to the isolated airborne pathogens. It is also worth mentioning that it is not definite that the admitted cases of respiratory diseases by the studied hospital belong exclusively to the studied zones. Therefore, precise correlations could not be made between the reported cases of respiratory diseases and the isolated microbial species during this study.

5. Conclusion

Eleven bacterial species and four fungal species were isolated and identified from the outdoor air environment of the four selected zone areas in Al-Mafraq governorate, Jordan. The levels of outdoor airborne bacteria and fungi in the open desert zone were found to be considerably lower than those found in Al-Mafraq downtown, Al al-Bayt University and Al-Zaatari refugee camp. It was also found that the isolated species does not correspond directly to the reported cases of respiratory diseases in this governorate. However, inhalation of or exposure to some of the isolated microorganisms might cause some human respiratory diseases. Therefore, implementation of better strategies for reducing the number of outdoor airborne bacteria and fungi would have benefits for human health.

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Some Records of Butterflies (Lepidoptera) from the Palestinian Territories

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Abstract

Butterflies were collected from 49 localities in the occupied West Bank of Jordan (Palestinian Territories). Fifty-four species were identified belonging to five families (Papilionidae, Pieridae, Lycaenidae, Hesperiidae, and Nymphalidae) during 2013-2015. The three most common butterflies observed and collected were the small White *Pieris rapae***,** the Bath White *Pontia daplidice*, and the Common Blue *Polyommatus icarus*. Many species seemed rare and to be threatened by loss of habitats including *Archon apollinus*, *Zegris eupheme*, *Gonepteryx cleopatra taurica*, and *Hipparchia fatua sichaea.* We suggest that the most significant threats to butterfly biodiversity in Palestine and the Arab World in general is habitat destruction and climate change.

Keywords: Lepidoptera, Palestine, Butterflies, Biodiversity, West Bank.

1. Introduction

Scientific studies on butterflies in Eastern Mediterranean started in early 20th century when many of the species and subspecies were described (Amsel, 1935a, 1935b, 1955; Amsel & Hering, 1931; Larsen, 1974; Larsen & Nakamura, 1983). [1] However, some of the older literature is problematical. For example, Bodenheimer (1935) provided dozens of anecdotal observations and speculations that are not substantiated by any data on the butterflies of Palestine. The work of Benyamini (1984, 1988, 1997, 2002a,b) focused on the areas of Palestine that became the state of Israel in 1949. The butterflies of Jordan were studied on different occasions (Larsen & Nakamura, 1983); the most recent updates include studies by Katbeh-Bader *et al*. (1998 [2003] & 2004); Saudi Arabia (Larsen, 1983; 1984); Lebanon (Larsen, 1974); and recently Egypt (Gilbert & Zalat, 2007). After the establishment of the Palestine Museum of Natural History in 2014, one of its obligations was to identify the neglected biodiversity elements of the West Bank, an area that has not been studied well by scientists since its occupation in 1967. The present study aims to identify the butterfly fauna of the West Bank (Occupied Palestinian Territory) and update its status.

2. Materials and Methods

All specimens were collected from the Occupied Palestinian Territory of the West Bank by The Palestine Museum of Natural History (PMNH) team. A total of 49 sampling stations were visited during 2013-2015 (Table 1). Butterflies were collected by means of nets and occasionally by hand from various localities and habitats. All collected specimens are deposited at PMNH.

The collected specimens were numbered and sexed. For each species, specimens were listed according to the alphabetical order of the collecting site and the date of collecting.

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[1] For more information about the history of studying Palestinian butterflies see Benyamini (1983).

Table 1. List of visited localities and their coordinates

Locality	N	E	Locality	N	E
Ain Al Fawar	31° 50'	35° 21'	Jericho	31° 51'	35° 27'
Ain Fara	31° 49'	35° 18'	Jiftlik	32°08’	35°29’
Ain Kenia	31° 55'	35° 9'	Jinsafut	32° 10	35° 07'
Ain Samiya	31° 59'	35° 20'	Kfr Zaybad	32° 13'	35° 4'
Ain Shibly - Bardala	32° 22'	35° 29'	Mar Saba.Bethlehem	31° 42'	35° 19'
Al Aqaba	32° 21'	35° 21'	Mikhmas	31° 52'	35° 16'
Al Aroub	31° 37'	35° 8'	Nabi Saleh	32° 0'	35° 7'
Al Nabi Mousa	31° 47'	35° 25'	Nahaleen	31° 41'	35° 7'
Al Qarn	31° 37'	35° 7'	Rawabi	32° 0'	35° 11'
Artas	31°69’	35°19’	Masafer Yatta	31° 26'	35° 7'
Auja	31°56′	35°27'	Salfit	32° 5	35° 10
Bardala	32° 23'	35° 28'	Silit Adahr	32° 22'	35° 19'
Beit Fajjar	31°37′	35°9'	Tayba	31° 56'	35° 18'
Beit Jala	31°42’	35°11’	Tarqumia	31° 34'	35° 1'
Beit Lid	32° 15'	35° 7'	Ubeidiya	31°43’	35°18’
Beit sahour	31°70’	35°22’	Umm Al Tut	32°25’	35°20’
Beni Neim	31° 31'	35° 9'	Wadi Fasayal	32°01′	35°26′
Bethlehem	31°42’	35°12’	Wadi Al Abyad, 6 km NW of Nuwaima	31°54’	35°23’
Birzeit	31° 58'	35° 11'	Wadi Al Qelt/Jericho	31° 50'	35° 24'
Bruqeen	32° 4’	35° 5’	Wadi Fukeen	31°71’	35°10’
Burak Sulaiman	31° 41'	35° 10'	Wadi Haramiya	31°59’	35°14’
Edhna	31° 33'	34° 58'	Wadi Quff	31° 33	34° 58'
Fasayil	32°01′	35°26′	Walaja	31° 43'	35° 9
Haris Village	32°06’	35°08’	Zeim, Jerusalem	32°01′	35°26′
Husan	31° 42'	35° 7'			

3. Results

Fifty-four species of butterflies belonging to five families (Papilionidae, Pieridae, Lycaenidae, Hesperiidae, and Nymphalidae) were identified.

Family Papilionidae

This family is represented in the Palestinian Territories by two subfamilies (Papilioninae and Parnassiinae) and three species.

Subfamily Papilioninae

Papilio machaon syriacus Verity, 1905 (Fig. 1-A) Swallowtail butterfly

Material examined: Beit Sahour (PMNH4445, ♂, 6.10.2014); Bethlehem (PMNH6355, ♂, 21.3.2015; PMNH4385, ♂, 6.8.2014; PMNH4506, ♂, 2.7.2014; PMNH5329, 2.7.2014; PMNH5330, ♀, 19.5.2014); Ubeidya (PMNH1748-7. ♂♂, 13.5.2013), Wadi Al Qelt (PMNH6201, ♀. 4.4.2015; PMNH6202, ♀, 4.4.2015; PMNH6204, ♀, 4.4.2015). Observed in Wadi Quf, Wadi Fukin, Battir, and Al Walaja.

Remarks: Specimens of this Holarctic butterfly were collected and observed during May to August, mostly in the southern part of the occupied West Bank where our studies concentrated. According to Benyamini (1997), these butterflies occur all year round, except for January, and are found throughout Palestine. It is found in nearby countries and it is not likely a migrant (Larsen, 1975). It feeds on members of the Apiaceae and Rutaceae, especially cultivated *Ruta graveolens* (Wiltshire, 1957; Larsen, 1974; Larsen & Nakamura, 1983; Katbeh-Bader *et al*., 1998 [2003]) and Cleomaceae (Halperin & Sauter, 1991-1992). Larvae are green banded with black and spotted with orange (Wiltshire, 1957). It is also considered as a pest that eats foliage of citrus trees, carrots, dill and fennel (Larsen, 1974).

Subfamily Parnassiinae

Archon apollinus (Herbst, 1798) False Apollo

Material examined: Bethlehem (PMNH5305, 16.12.2014; PMNH5593, 21.1.2015; PMNH5663, 30.1.2015; PMNH5668, 30.1.2015).

Remarks: The genus *Archon* is found only in areas of the Eastern Mediterranean extending from Turkey to Iran, with Palestine being the most southern range of the distribution (Larsen & Nakamura, 1983). Specimens were collected from Bethlehem (PMNH garden) in February; it was also observed in March. Benyamini (1997) collected them during late November till April. According to Katbeh-Bader *et al.* (1998 [2003]), this species is limited to Bulgaria, Turkey, the Levant and Iraq. Larvae are black and spotted with orange dots and feeds on all species of *Aristolochia* (Larsen & Nakamura, 1983). The likely subspecies of our material is *A. a. bellargus* Staudinger, 1891. Nazari & Sperling (2007) found a significant genetic divergence between populations from Palestine and those from Turkey, suggesting that there might be more species in this complex than what was reported previously. *Archon apollinus* is nearly threatened in Europe according to the IUCN criteria (Van Swaay *et al.*, 2011).

Allancastria deyrollei eisneri (Bernardi, 1971) (Fig. 1-B) The Lebanese Festoon

Material examined: Bethlehem (PMNH6409, ♀, 27.3.2015).

Remarks: According to Benyamini (1983) *A. deyrollei* was recorded for the first time in Palestine in 1983, and inhabits the northern and central region of Palestine. This species is likely to ascend up to 2000m (Larsen, 1974). A single specimen was collected from Bethlehem below 800m at the southern end of its known distribution in Palestine, but it seems to penetrate the southern Mediterranean zone in Jordan (Larsen & Nakamura, 1983). The Lebanese Festoon is known to feed on various species of the family Aristolochiaceae, such as *Aristolochia scabridula* and *Aristolochia maurorum* (Larsen, 1974). *A. deyrollei* is widespread in Turkey, the Levant, and Iran with a single brood from March to April (Katbeh-Bader *et al.*, 1998 [2003]; Nazari *et al.*, 2007) and the larvae usually pupate under stones in rudimentary cocoons (Larsen & Nakamura, 1983).

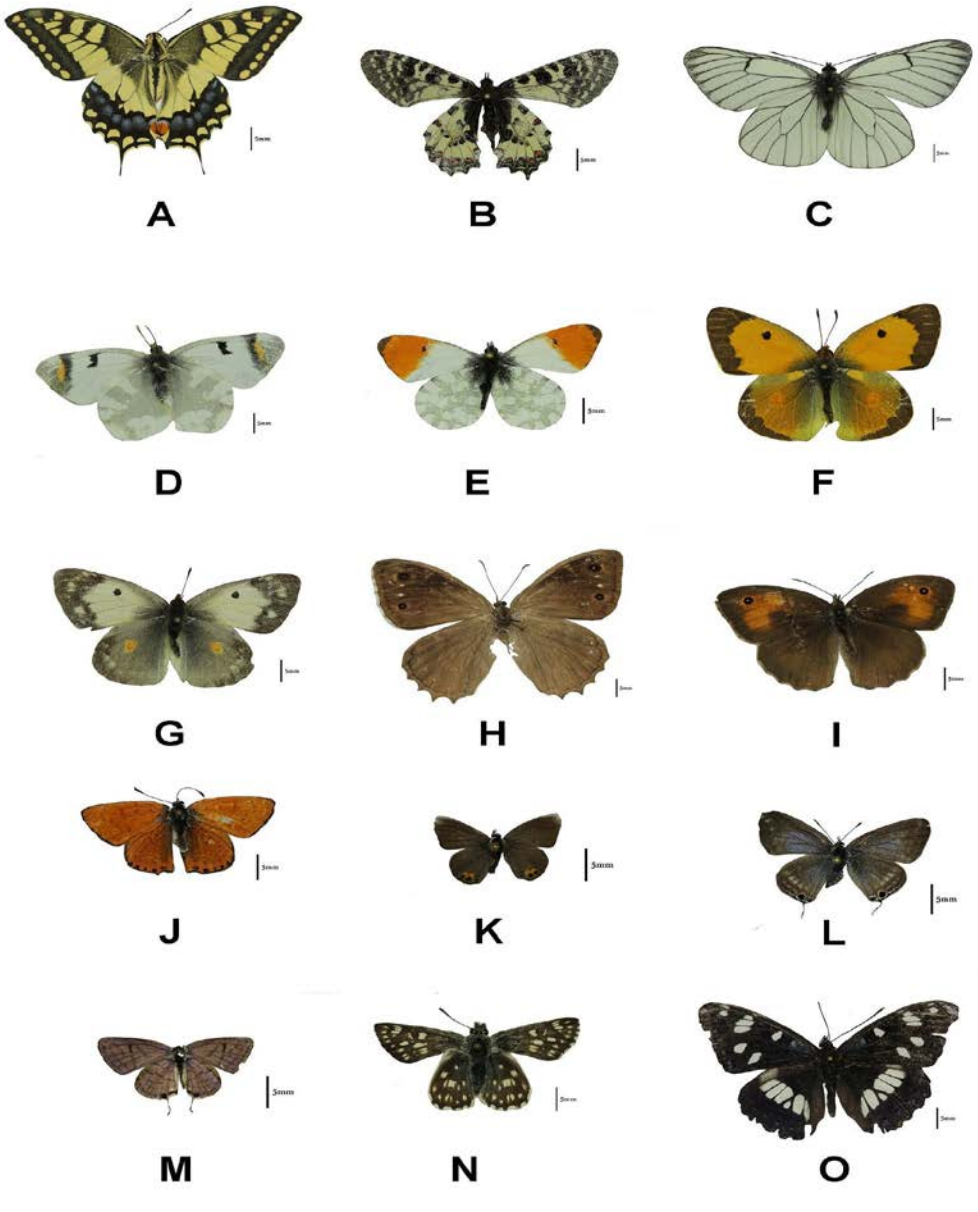

Figure 1. A- *Papilio machaon syriacus*, B- *Allancastria deyrollei eisneri*, C- *Aporia crataegi augustior,* D- *Zegris eupheme,* E- *Anthocharis cardamines phoenissa,* F- *Colias croceus,* G- *Colias croceus* morph, H- *Hipparchia pisidice, I- Maniola telmessia,* J- *Lycaena thersamon,* K- *Chilades (Freyeria) trochylus,* L- *Lampides boeticus,* M- *Tarucus rosaceus,* N- *Syrichtus tessellum nomas,* O- *Limenitis reducta schiffermuelleri.*

Family Pieridae

Based on the updated classification, there are four subfamilies and only two occur in Palestine (Pierinae and Coliadinae).

Subfamily Pierinae

Anaphaeis aurota **(**Fabricius, 1793) Brown-veined White

Material examined: Ain Fasayel (PMNH5500, ♂, 14.1.2015); Artas (PMNH5251, ♂, 12.8.2014); Beit Sahour (PMNH4092, ♀, 4.5.2014); Bethlehem (PMNH6440, ♂, 20.4.2015; PMNH6352, ♀, 25.10.2014; PMNH4988, ♂, 17.8.2014; PMNH5300, ♂, 8.11.2014); Edna (PMNH4996, ♂, 23.8.2014; PMNH4999, ♂, 23.8.2014; PMNH5002, ♂, 23.8.2014; PMNH5004, ♀, 23.8.2014; PMNH5010, ♂, 23.8.2014); Salfit (PMNH5074, ♂, 22.8.2014; PMNH5093, ♀, 22.8.2014); Wadi Al Qelt (PMNH6197, ♂, 4.4.2015).

Remarks: Specimens were collected in January, April, May, August, October, and November. Larsen (1975) and Benyamini (1983) reported that this species declined in both Palestine and Lebanon since the 1940s and 1950s. However, it seems to recover in certain areas. Brown-veined White butterfly is known in Africa, Arabia and India (Larsen, 1990). Larvae feed on *Capparis* sp. and *Maerua* sp. (Larsen, 1990).

Anthocharis cardamines phoenissa von Kalchberg, 1894 (Fig. 1-E) Orange Tip

Material examined: Bethlehem (PMNH5688, ♂, 8.2.2015; PMNH6408, ♀, 14.3.2015; PMNH6407, ♂, 16.3.2015; PMNH6406, ♀, 10.3.2015; PMNH6396, ♀, 24.3.2015; PMNH63594, ♂, 21.3.2015; PMNH6389, ♂, 26.3.2015; PMNH6387, ♂, 26.3.2015; PMNH6386, ♂, 27.3.2015; PMNH6380, ♀, 26.3.2015; PMNH6379, ♀, 26.3.2015; PMNH6354, ♂, 24.2.2015; PMNH6351, ♀, 24.2.2015; PMNH6028, ♂, 26.2.2015); Wadi Al Qelt (PMNH6417, ♀, 28.3.2015; PMNH6418, ♂, 17.3.2015).

Remarks: Collected in February and March. *A. cardamines* is common in Western Europe, temperate Asia to China (Larsen, 1974). In Palestine, Orange Tip is common in the coastal and central areas (Benyamini, 1983). It has a single brood that appears in late February and the larva feeds on various Brassicaceae, including *Torilis* sp., *Alliaria* sp., *Cardamine* sp., *Barbarea* sp., *Cochlearia* sp. and *Nasturtium* sp.

Aporia crataegi augustior Graves, 1925 (Fig. 1-C) The Black-veined White

Material examined: Al Makhrour-Bethlehem (PMNH6326, ♂, 19.4.2015; PMNH6329, ♂, 15.4.2015), Wadi Al Qelt (PMNH6323, ♂, 2.4.2015; PMNH6324, ♂, 2.4.2015), Wadi Fukein (PMNH6318, ♂, 19.4.2015).

Remarks: The Black-veined White is found from North Africa, Western Europe to the Middle East and the Far East. Our specimens were collected in April. This spring species is found in our area from March to June (Benyamini, 1997). According to Benyamini (1983), this butterfly is common in north and central Palestine; excluding Jericho; but we also caught it at Wadi Al Qelt (Jericho area). *A. crataegi augustior* feeds on Rosaceae such as *Crataegus* sp. and *Amygdalus* sp. (Halperin & Sauter, 1992).

Colotis fausta fausta **(**Olivier, 1804) Large Salmon Arab

Material examined: Ain Fara (PMNH4978, ♂, 16.8.2014); Ain Kenia (PMNH4794, ♂, 15.8.2014; PMNH4796, ♂, 15.8.2014; PMNH4802, ♂, 15.8.2014); Al Walaja (PMNH4609, ♀ , 8.8.2014; PMNH4612, ♂, 8.8.2014; PMNH4616, ♀, 8.8.2014; PMNH4635, ♂, 8.8.2014; PMNH4675, ♂, 11.8.2014; PMNH5250, ♂, 20.9.2014); Artas (PMNH4730, ♂, 13.8.2014; PMNH4731, ♂, 13.8.2014); Bethlehem (PMNH5341, 2.7.2014; PMNH5342, ♂, 22.6.2014; PMNH5343, 8.8.2014; PMNH4596, ♂, 22.7.2014; PMNH4740, ♀, 13.8.2014); Edna (PMNH4989, ♀, 23.8.2014); Mar Saba-Bethlehem (PMNH5935, ♂, 13.3.2015); Burak Sulaiman (PMNH4963, ♂, 17.8.2014);; Salfit (PMNH5046, ♂, 22.8.2014; PMNH5046, ♂, 22.8.2014; PMNH5047, ♂, 22.8.2014; PMNH5057, ♂, 22.8.2014; PMNH5067, ♂, 22.8.2014; PMNH5070, ♂, 22.8.2014; PMNH5072, ♂, 22.8.2014; PMNH5085, ♀, 22.8.2014; PMNH5086, ♀, 22.8.2014; PMNH5087, ♀, 22.8.2014; PMNH5097, ♂, 22.8.2014; PMNH5101, ♂, 22.8.2014); Wadi Al Quff (PMNH3000-8, ♂♂, 30.8.2013); Wadi Fukein (PMNH4697, ♂, 9.8.2014).

Remarks: Specimens were collected between March and September. *M. fausta* is common in the Mediterranean zone, the Arabian Peninsula, India and Iraq (Larsen & Nakamura, 1983). In Palestine, it was collected even from arid areas (near the Dead Sea) all year round (Benyamini, 1997). Larvae feed on *Capparis* sp., and *Cartilaginea* (Larsen, 1990; Halperin & Sauter, 1992).

Euchloe ausonia melisande Fruhstorfer, 1908 The Dappled White

Material examined: Bethlehem (PMNH6360, ♀, 28.3.2015; PMNH6362, ♂, 26.3.2015; PMNH6369, ♀, 16.3.2015; PMNH6372, ♀, 14.3.2015; PMNH6405, ♂, 24.3.2015; PMNH5957, ♂, 13.3.2015); Mar Saba (PMNH5911, ♂, 13.3.2015); Wadi Al Qelt (PMNH6420, ♂, 17.3.2015).

Remarks: All specimens of this species were collected in March. The *E. ausonia* complex is found all around the Mediterranean and in Asia Minor. The Dappled White is common in both Mediterranean zones of Jordan. It feeds on *Brassica* and *Sinapis* (Brassicaceae). It was collected from several localities along the Jordanian side of the Jordan Valley (Katbeh-Bader *et al*., 2003).

Euchloe belemia belemia **(**Esper, 1799) The Green-striped White

Material examined: Bethlehem (PMNH6388, ♂, 26.3.2015; PMNH5667, ♀, 3.1.2015); Mar Saba (PMNH5918, ♂, 13.3.2015); Auja (PMNH5885, ♀, 9.3.2015; PMNH5887, 9.3.2015; PMNH5888, 9.3.2015; PMNH5886, 9.3.2015); Al Nabi Mousa (PMNH5940, 13.3.2015); Matahen Al Sukkar (PMNH5547, ♂, 21.1.2015); Wadi Al Qelt (PMNH6416, ♂, 24.2.2015).

Remarks: Specimens were collected in January in the Jordan Valley around Jericho and in March in the Bethlehem area. The Green-striped White extends from Iberian Peninsula, via North Africa to the Middle East and Iran to Baluchistan. In addition, it was recorded in Ethiopia and Arabia. It is a common species in the northern Mediterranean zone of Jordan and known to occur in the Jordan Valley (Katbeh-Bader *et al*., 2003).

Apparently, it has one brood in the spring, with highest peak of emergence in April. It feeds on *Erucaria* in the Jordan Valley (Trought, in Larsen & Nakamura, 1983).

Euchloe charlonia **(**Donzel, 1842) Greenish Black-tip

Material examined: Ain Hijla (PMNH 3992, 18.4.2014), Bethlehem (PMNH6384, ♂, 9.3.2015; PMNH6383, ♀, 9.3.2015; PMNH 5304, ♀, 16.12.2014); Jericho (PMNH5429, ♂, 12.1.2015); Mar Saba (PMNH5963, ♂, 13.3.2015; PMNH5966, ♂, 13.3.2015; PMNH5961, ♂, 13.3.2015; PMNH5960, ♀, 13.3.2015;PMNH 5951, ♂, 13.3.2015; PMNH5915,♂, 13.3.2015; PMNH5936, ♂, 13.3.2015; PMNH5949,♀, 13.3.2015; PMNH5945, ♂, 13.3.2015); Wadi Al Qelt (PMNH5897, ♀, 9.3.2015; PMNH5896, ♂, 9.3.2015); Wadi Al Quff (PMNH5549, ♂, 24.1.2015).

Remarks: Specimens were collected in January, March, April and December. Our data are in agreement with Larsen & Nakamura (1983) that the Greenish Black-tip has two broods: One in March and the second in November. This species is known from Morocco to Western India but its distribution is rather sporadic. *E. charlonia* is common in Hebron to Tiberias except coastal areas (Benyamini, 1997). The larval food plants are *Diplotaxis* sp., *Lonchophora* sp. and other Brassicaceae.

Pieris brassicae **(**Linnaeus, 1758) Large cabbage white

Material examined: Ain Fasayel (PMNH5465, ♀, 14.1.2015); Al Makhrour-Bethlehem (PMNH6539, ♀, 3.5.2015); Bethlehem (PMNH6346, ♂, 24.2.2015; PMNH6357, ♀, 16.3.2015; PMNH5320, ♀, 18.6.2014; PMNH5321, ♀, 15.8.2013); Burak Sulaiman (PMNH5749, 16.2.2015); Jiftlik (PMNH1708-12, ♂♂, 27.3.2013); Mar Saba-Bethlehem (PMNH5920, ♂, 13.3.2015; PMNH5937, ♀, 13.3.2015; PMNH5947, ♂, 13.3.2015; PMNH5926, ♀, 13.3.2015); Wadi Al Qelt (PMNH6198, ♂, 4.4.2015); Wadi Al Quff (PMNH3965, ♀, 11.4.2014; PMNH3967, ♂, 4.11.2014); Wadi Qana (PMNH5612, ♂, 26.11.2014).

Remarks: Specimens were collected during January and August, with three specimens collected in November. *P. brassicae* is associated with many cultivated fields including cabbage and other vegetables around human habitation and is not migratory in our region (Larsen, 1975). There are slight variations in wing patterns in this species (Freitak *et al.*, 2005). This Palaearctic species is common throughout North Africa, Europe, and Eastern Mediterranean regions with more than one successful brood (Larsen, 1974). Benyamini (1997) and Katbeh-Bader *et al.* (2004) reported this species all year round, except for January; however, we collected specimens during January from the Jordan Valley at Ain Fasayel where the climate is warm all year round. It mainly feeds on family Brassicaceae and Capparaceae (Halperin & Sauter, 1992; Katbeh-Bader *et al*., 2004). Subspecies commonly assigned to our material is *catoleuca* Röber 1896 but there are many issues in subspecies designations and we prefer not to use.

Pieris rapae leucosoma **(**Schawerda, 1905) The Small White

Material examined: 3 km W Mar Saba (PMNH5950, ♂, 13.3.2015; PMNH5921, ♂, 13.3.2015; PMNH5912, ♂, 13.3.2015); Ain Hijla (PMNH4002, ♂, 18.4.2014; PMNH 4000, ♂, 18.4.2014; PMNH4001, ♂, 18.4.2014; PMNH4024, ♂, 18.4.2014); Ain Kenia (PMNH4811, ♀, 15.8.2014); Ain Shibly-Bardala (PMNH6554, ♂, 6.5.2015); Al Aroub (PMNH4588, ♂, 7.7.2014; PMNH4589, ♂, 7.7.2014); Al Makhrour-Bethlehem (PMNH6543, ♀, 3.5.2015; PMNH6325, ♂, 15.4.2015); Al Walaja (PMNH4618, ♀, 8.8.2014); Artas (PMNH4718, ♂, 12.8.2014); Auja (PMNH1710-15, ♂♂, 27.3.2013; PMNH1710-11, ♂♂, 27.3.2013; PMNH5884, ♂, 9.3.2015); Bethlehem (PMNH5428, ♂, 27.4.2015; PMNH6349, ♂, 24.2.2015; PMNH6348, ♂, 24.2.2015; PMNH6393, ♂, 26.3.2015; PMNH6390, ♂, 26.3.2015; PMNH6398, ♀, 13.3.2015; PMNH6400, ♂, 16.3.2015; PMNH6401, ♂, 26.3.2015; PMNH6402, ♂, 26.3.2015; PMNH6403, ♂, 21.3.2015; PMNH6399, ♂, 21.3.2015; PMNH6404, ♂, 14.3.2015; PMNH6391, ♂, 16.3.2015; PMNH6392, ♂, 16.3.2015; PMNH6397, ♀, 21.3.2015; PMNH6395, ♂, 21.3.2015; PMNH5889, ♂, 16.3.2015; PMNH4508, ♂, 2.7.2014; PMNH5306, ♀, 10.5.2014; PMNH5307, ♀, 10.5.2014; PMNH5308, ♂, 10.5.14; PMNH4329, ♂, 6.2.2014); Beit Qad (PMNH6066, ♂, 18.3.2015; PMNH6067, ♂, 18.3.2015; PMNH6068, ♂, 18.3.2015); Bruqeen (PMNH5165, ♂, 22.8.2014); Burak Sulaiman (PMNH4964, ♀, 17.8.2014); Edna (PMNH4997, ♂, 23.8.2014; PMNH5000, ♂, 23.8.2014; PMNH5005, ♂, 23.8.2014); Em El Tout (PMNH6548, ♂, 6.5.2015); Kfr Zaybad (PMNH1755-20, ♂♂, 18.5.2013; PMNH1755-28, ♂♂, 18.5.2013); Mar Saba-Bethlehem (PMNH5932, ♂, 13.3.2015; PMNH5953, ♀, 13.3.2015); Mikhmas (PMNH 1759-9, ♀♀, 23.5.2013); Salfit (PMNH5044, ♂, 22.8.2014; PMNH5051, ♂, 22.8.2014; PMNH5075, ♂, 22.8.2014; PMNH5077, ♂, 22.8.2014; PMNH5079, ♂, 22.8.2014; PMNH5084, ♀, 22.8.2014); Tulkarm (PMNH6069, ♂, 19.3.2015; PMNH6070, ♂, 19.3.2015; PMNH6071, ♂, 19.3.2015; PMNH6072, ♂, 19.3.2015); Wadi Al Abyad, 6 km NW of Nuwaima (PMNH6500, ♂, 24.4.2015); Wadi Al Quff (PMNH4079, ♀, 21.4.2014; PMNH4448, ♀, 6.9.2014; PMNH5303, ♂, 30.11.2014); Wadi Al Qelt/Jericho (PMNH6195, ♂, 4.4.2015; PMNH6196, ♂, 4.4.2015; PMNH6194, ♀, 4.4.2015; PMNH6193, ♀, 4.4.2015; PMNH6192, ♂, 4.4.2015; PMNH5900, ♀, 9.3.2015; PMNH5905, ♀, 9.3.2015; PMNH6415, ♂, 9.3.2015; PMNH6419, ♂ 28.2.2015; PMNH5903, ♀, 9.3.2015); Wadi Fukein (PMNH6314, ♀, 19.4.2015); Wadi Haramiya (PMNH1730-1, ♂♂, 18.4.2014; PMNH 4111, ♂, 15.5.2014).

Remarks: This was one of the most common species we encountered with specimens collected during most months of the year from most localities visited during the present study. Migrations in this species are well documented, including over sea water in the Eastern Mediterranean region (John *et al*., 2008). *P. rapae* is found in the Mediterranean zone, including the Levant and Egypt. It is common in all Palestine, except for the Negev all year round (Benyamini, 1997). Larvae feed on Cruciferae like cabbage, and the adult butterflies prefer to take the nectar from flowers of alfalfa, *Medicago sativa* (Larsen, 1990). Other plants are associated with this species, including the families Capparaceae (Halperin & Sauter, 1992).

Pontia daplidice **(**Linnaeus, 1758) Bath White

Material examined: 3 km w Mar Saba (PMNH5990, ♂, 13.3.2015); Mar Saba-Bethlehem (PMNH5955, ♀, 13.3.2015; PMNH5948, ♀,13.3.2015; PMNH5928, ♀.13.3.2015; PMNH5938, ♀, 13.3.2015; PMNH 5925, ♀.13.3.2015; PMNH5923, ♀, 13.3.2015; PMNH5917, ♀, 13.3.2015; PMNH5916, ♀, 13.3.2015; PMNH5914, ♀, 13.3.2015); Ain Hijla (PMNH3988, ♂, 18.4.2014; PMNH3990, ♂, 18.4.2014; PMNH4010, ♀, 18.4.2014; PMNH4011, ♂, 18.4.2014); Ain Fasayel (PMNH5460, ♀, 14.1.2015); Ain Kenia (PMNH4808, ♂, 15.8.2014); Al Aqaba (PMNH 4044, ♂, 18.4.2014); Al Makhrour-Bethlehem (PMNH6310, ♀, 15.4.2015; PMNH6327, ♂, 15.4.2015; PMNH6330, ♂, 15.4.2015; PMNH6335, ♀, 15.4.2015); Al Nabi Mousa (PMNH5941, ♀, 13.3.2015); Al Shawawreh-Bethlehem (PMNH5395, ♂, 26.12.2014); Al Walaja (PMNH4661 ♀, 8.8.2014); Artas (PMNH4732, ♀, 13.8.2014); Beit Lid (PMNH3469, ♀, 1.2,2014); Beirzeit (PMNH3974, ♂, 15.4.2014; PMNH3969, ♀, 15.4.2014); Bethlehem (PMNH6359, ♀, 23.3.2015; PMNH6381, ♂. 10.3.2015; PMNH6382, ♀, 10.3.2015; PMNH5939, ♀, 13.3.2015; PMNH5317, ♀, 14.5.2014); Burak Sulaiman (PMNH5705, ♀, 16.2.2015); Safeer (PMNH1713, ♀, 7.4.2013); Salfit (PMNH4094, ♂, 22.8.2014; PMNH5082, ♂, 22.8.2014; PMNH5098, ♂, 22.8.2014; PMNH5099, ♀, 22.8.2014); Wadi Al Quff (PMNH3871, ♀, 16.3.2014; PMNH3964, ♀, 11.4.2014; PMNH3912, ♂, 21.3.2014; PMNH3941, ♀, 4.11.2014; PMNH3942, ♀, 4.11.2014; PMNH3945, ♀, 4.11.2014; PMNH 3949, ♀, 4.11.2014; PMNH4026, ♂, 21.4.2014; PMNH4028, ♀, 21.4.2014; PMNH4033, ♂, 21.4.2014; PMNH4034, ♀, 21.4.2014; PMNH4076 ♀, 21.4.2014; PMNH4077, ♂, 21.4.2014; PMNH4102, ♀, 5.3.2014; PMNH4331, ♀, 5.3.2014; PMNH4342, ♀, 5.3.2014; PMNH4451, ♂, 6.9.2014; PMNH4452, ♀, 6.9.2014); Wadi Al Qelt - Jericho (PMNH6200, ♂, 4.4.2015; PMNH6206, ♂, 4.4.2015; PMNH5766, 23.2.2015; PMNH5881 ♀, 9.3.2015; PMNH5823, ♀, 9.3.2015; PMNH5901, ♀, 9.3.2015; PMNH5838, ♀, 9.3.2015; PMNH5835, ♀ , 9.3.2015; PMNH5899, ♀, 9.3.2015; PMNH5833, ♀, 9.3.2015; PMNH5904, ♀, 9.3.2015; PMNH5830, ♀, 9.3.2015; PMNH5902, ♀, 9.3.2015; PMNH5877, ♀, 9.3.2015; PMNH5837, ♀, 9.3.2015; PMNH5898, ♀, 9.3.2015; PMNH5906, ♀, 9.3.2015); Wadi Fukein (PMNH6313, ♂,19.4.2015; PMNH6322, ♂, 19.4.2015).

Remarks: Recently, this complex has been noted to possibly include two species or two major subspecies that are almost identical morphologically but differing at the molecular level: *P. daplidice* and *P. edusa* (Geiger & Scholl, 1982; John *et al*., 2013). This seems to be a very common and widely distributed species throughout Palestine. Like other authors (Benyamini, 1983, 1997; Larsen, 1975 & 1982), we collected specimens of this migratory species throughout the year.

Pontia glauconome glauconome (Klug, 1829) Desert White

Material examined: 3 km w Mar Saba (PMNH5991, ♀, 13.3.2015); Ain Al Fawar (PMNH 4984, ♀, 16.8.2014); Ain Hijla (PMNH 3991, ♀, 18.4.2014; PMNH4012, ♂, 18.4.2014); Ain Kenia (PMNH4832, ♀, 15.8.2014); Al Qarn (PMNH4585, ♂, 7.7.2014); Al Walaja (PMNH4631, ♀, 8.8.2014); Bethlehem (PMNH5319, ♀, 10.5.2014; PMNH5318, ♂, 15.8.2013); Salfit (PMNH5094, ♂, 22.8.2014); Wadi Al Quff (PMNH4447, ♀, 6.9.2014; PMNH4330, ♀, 5.3.2014; PMNH4449, ♂, 6.9.2014; PMNH4081, ♀, 5.3.2014; PMNH 4454, ♂, 6.9.2014; PMNH 4450, ♂, 6.9.2014).

Remarks: Collected from March to September, except in June. According to Benyamini (1997), *P. glauconome* is found all year round, except in January. This is a Saharo-Sindian and eremic species, with a known distribution in North Africa, Jordanian desert, Sinai Peninsula, Iraq, Oman and in Palestine where it is common in the Dead Sea area and the Negev (Benyamini, 1983 & 1997; Larsen & Larsen, 1980; Larsen, 1990). However, we observed it in the Mediterranean areas, including records from Bethlehem, Wadi Al Quff, and Salfit. Food plants of this species include the families Capparicaceae and Resedaceae (Halperin & Sauter, 1992).

Zegris eupheme **(**Esper, 1804) (Fig. 1-D) The Sooty Orange Tip

Material examined: Wadi Al Qelt (PMNH5832, ♀, 9.3.2015).

Remarks: A single specimen was collected in March from Wadi Al Qelt, Jericho. According to Larsen & Nakamura (1983), *Z. eupheme* is found in the Irano-Turanian zone from the dry part of Spain, Morocco and to the desert between Jordan and Iraq. Locally, the subspecies *urda* Hemming, 1929 seems to appear in the Dead Sea area and the northern parts of Negev (Benyamini, 1983 & 1997). The Sooty Orange Tip is found in one brood in early spring. It feeds mainly on the family Brassicaceae, especially *Isatis tinctoria* and *Erucaria boveana* and other Cruciferae may host larvae (Courtney, 1982; Larsen & Nakamura, 1983). This species is nearly threatened in Europe per IUCN criteria (Van Swaay *et al*., 2011); we have only one specimen, which may suggest that it is threatened here in Palestine.

Subfamily Coliadinae

Colias croceus **(Fourcoy, 1785)** (Fig. 1-F and G) Clouded Yellow

Material examined: Al Makhrour–Bethlehem (PMNH538, ♀, 3.5.2015; PMNH6328, ♂, 15.4.2015; PMNH6300, ♂, 15.4.2015); Auja (PMNH 5851, ♂, 9.3.2015; PMNH5850, ♂, 9.3.2015); Bethlehem (PMNH6436, ♀, 20.4.2015; PMNH6410, ♀, 28.3.2015; PMNH6356, ♀, 26.3.2015); Edna (PMNH4987, ♀, 23.8.2014; PMNH4990, ♀, 23.8.2014; PMNH4994, ♂, 23.8.2014; PMNH4995, ♂, 23.8.2014); Salfit (PMNH5078, ♂, 22.8.2014); Nabi Saleh (PMNH1736-22, ♂♂, 3-4.5.2013), Wadi Al Qelt (PMNH5831, ♀, 9.3.2015; PMNH6199, ♂, 4.4.2015); Wadi Haramiya (PMNH4109, ♂, 15.5.2014).

Remarks: Specimens were collected in March and August. *Colias* as a genus (clouded yellow butterflies) has three species that potentially occur in Palestine: *C. libanotica* Lederer 1858, *C. croceus* Geoffroy 1785 and *C. erate* Esper, 1805. Recent molecular studies have been performed on *Colias*, showing that the sister taxon for *C. croceus* is *C. erate* (Pollock *et al*., 1998). The latter is mentioned in Lebanon and Cyprus although the records from Cyprus maybe misidentified and are actually *croceus*. Other color forms can be observed for this species even within the same location (Fig. 1-G).

Gonepteryx cleopatra taurica (Staudinger, 1881) The Cleopatra

Material examined: Bethlehem (PMNH5686, ♂, 6.2.2015).

Remarks: A single sample was collected from Bethlehem in February. According to Benyamini (1997), *G. cleopatra* can be observed all year round except in December. The subspecies present in Palestine is *taurica* which is ponto-mediterranean found in Turkey and the Levant (Larsen, 1983). *G. cleopatra* is found in northern to the middle of Palestine, except for the Jordan Valley (Benyamini, 1997). It is a migratory species and feeds on *Rhamnus* sp. (Larsen, 1974 & 1983). Decline in its numbers and distribution may reflect the degradation of forests (Katbeh-Bader *et al*., 2003).

Subfamily Satyrinae

Hipparchia fatua sichaea (Lederer, 1857) The Freyer's Grayling

Material examined: Wadi Fukeen (PMNH4693, ♂, 9.8.2014).

Remarks: One specimen was collected in August. *H. fatua* is common throughout Balkans and Middle East to Iran and Turkmenistan (Larsen & Nakamura, 1983). In Palestine, it is widespread in the northern and middle areas (Benyamini, 1983) around well-wooded regions, such as Wadi Fukeen. The Freyer's Grayling appears from early summer till late autumn and feeds on grasses according to Larsen & Nakamura (1983) but this is a rare species that is likely threatened in our area by development. Other species of the genus are threatened or nearly threatened in Europe (Van Swaay *et al*., 2011).

Hipparchia pisidice Klug, 1932 (Fig. 1-H) The Sinai Grayling

Material examined: Salfit (PMNH5062, ♀, 22.8.2014); Wadi Fukeen (PMNH5143, ♀, 29.8.2014).

Remarks: We collected specimens in August. The Sinai Grayling occurs in Sinai, the Levant and southern parts of Turkey (Katbeh-Bader *et al*., 2003). It was previously recorded in several localities in the northern Mediterranean zone of Jordan only. Larvae feed on grasses.

Hyponephele lupinus centralis (Riley, 1921) The Oriental Meadow Brown

Material examined: Beit Fajjar (PMNH5118, ♀, 23.8.2014; PMNH5120, ♀, 23.8.2014).

Remarks: Specimens were collected in August. The Oriental Meadow brown occurs in North Africa, southern Europe, Asia Minor, the Levant, Iran, Afghanistan (Katbeh-Bader *et al*., 2003). It has a single brood in May and June or July. Specimens collected in August or September are assumed to be aestivating individuals appearing to oviposit (Larsen & Nakamura, 1983). Larvae feed on grasses.

Lasiommata maera (Linnaeus, 1758) The Large Wall Brown

Material examined: Al Walaja (PMNH4592, 19.8.2014); Bethlehem (PMNH6365, 28.3.2015; PMNH5322, ♂, 3.7.2014; PMNH5323, ♂, 3.7.2014; PMNH5324, 22.5.2014; PMNH5325, ♂, 18.5.2014; PMNH5326, ♂, 18.5.2014;PMNH 5327, ♂, 11.5.2014; PMNH4507, ♂, 2.7.2014;PMNH5328, ♂, 9.8.2013); Birzeit (PMNH1729-1, ♂♂, 4.11.2013); Haris village (PMNH4594, ♀, 1.7.2014; PMNH4593, ♂, 1.7.2014); Mikhmas (PMNH1759-3, 23.5.2013); Wadi Al Quff (PMNH3872, ♀, 16.3.2014; PMNH3959, ♂, 4.11.2014; PMNH3000-10, ♀♀, 30.8.2013; PMNH3771, 15.3.2014; PMNH4333, 5.3.2014).

Remarks: Specimens were collected in March, May, July, August and November. The Large Wall Brown has a distribution that extends from North Africa, Europe, and the Middle East to Central of Asia. In Palestine, *L. maera* ranges from northern Palestine to Hebron in the south (Benyamini, 1983). We noted a great variation in our samples, so we do not prefer to use the ssp. *orientalis* Heyne, 1894, which is used for the Levantine populations. Collecting dates suggests that this butterfly has three broods as Larsen & Nakamura (1983) expected for the Jordanian population, two of them in early spring and summer and the third in September. Larvae feed on grasses including *Poa, Glyceria, Hordeum* and *Festuca* (Larsen & Nakamura, 1983).

Lasiommata megera emilyssa (Verity, 1919) The Wall Brown

Material examined: Umm El Tout (PMNH6549, ♂, 6.5.2015).

Remarks: A single specimen was collected in May. *L. megera* is found in North Africa, Europe and the Middle East to Iran. In Palestine, the Wall Brown is widespread from northern Palestine to the Negev borders. Like *L. maera*, Larsen & Nakamura (1983) suggested three broods for *L. megera* from February to September. Obviously, it is less abundant than the *L. maera* species.

Maniola telmessia (Zeller, 1847) (Fig. 1-I) The Eastern Meadow Brown

Material examined: Jerusalem (PMNH1749-5, ♀♀, 13.5.2013); Nahaleen (PMNH1735-16, ♂♂, 5.2.2013; PMNH1735-8, ♀♀, 5.2.2013); Tarqurija (PMNH1712-4, ♂♂, 4.4.2013); Wadi Fukeen (PMNH6317, ♂, 19.4.2015; PMNH6324, ♂, 19.4.2015); Wadi Al Quff (PMNH3000-3, ♀♀, 30.8.2013; PMNH4030, ♂, 21.4.2014; PMNH4032, ♂, 21.4.2014; PMNH4460, ♀, 6.9.2014).

Remarks: We collected specimens in Mediterranean habitats during February, April, August, and September. The Eastern Meadow Brown is found in Turkey, Iran and the Levant. It is restricted to the northern Mediterranean zone. It has one brood in April and May (Katbeh-Bader *et al*., 2003). Specimens collected later in the year are aestivating individuals appearing to oviposit (Larsen & Nakamura, 1983). The *Maniola* group has undergone recent speciation and had likely migrated during the last glacials into the Levant (Kreuzinger *et al*., 2015).

Melanargia titea (Klug, 1832) The Levantine Marbled White

Material examined: Ain Samiya (PMNH1731-13, ♀♀, 12.4.2013; PMNH1731-9, ♀♀, 12.4.2013); Bethlehem (PMNH6439, ♀, 20.4.2015; PMNH6438, ♀, 20.4.2015; PMNH5338, 1.5.2014; PMNH5339, 11.5.2014); Beni Neim (PMNH1714-27, ♂♂, 7.4.2013); Birzeit (PMNH1729-2, ♀♀, 11.4.2013; PMNH1729-3, ♂♂, 11.4.2013; PMNH1729-4, ♀♀, 11.4.2013); Mikhmas (PMNH1733-1, ♀♀, 27.4.2013); Nahaleen (PMNH1735-3, ♂♂, 2.5.2013; PMNH1735-4, ♂♂, 2.5.2013); Rawabi

(PMNH1732,♀, 18.4.2013); Masafer Yatta (PMNH1713-8, ♂♂, 7.4.2013; PMNH1713-9, ♂♂, 7.4.2013); Tayba (PMNH1734-23, ♀♀, 12.4.2013); Wadi Fasayel (PMNH4075, ♂, 21.4.2014); Wadi Fukein (PMNH6315, 19.4.2015; PMNH6316, 19.4.2015; PMNH6319, ♂, 19.4.2015; PMNH6320, 19.4.2015); Wadi Haramiya (PMNH4106, ♀, 15.5.2014; PMNH4107, ♀, 15.5.2014; PMNH4108, ♂, 15.5.2014).

Remarks: The Levantine Marbled White is limited to the Mediterranean region from southern Turkey to far south Jordan (Larsen & Nakamura, 1983). In Palestine, *M. titea* has two subspecies: *titania* and *palaestinensis* which were observed in the northern part of Palestine with Beni Niem being the most southern locality. We collected specimens in April and May.

Pseudochazara thelephassa (Geyer, [1827]) The Telephassa Grayling

Material examined: Mikhmas (PMNH1759-1, ♀♀, 23.5.2013); Walaja (PMNH4636, ♀, 8.8.2014).

Remarks: Specimens were collected in May and August. This species migrates in Turkey (Osthelder & Pfeiffer, 1932) and in Lebanon (Larsen, 1975). Even though it was collected from June to August, Larsen & Nakamura (1983) mentioned records in October and they assumed a single protracted brood.

Ypthima asterope (Klug, 1832) The African Ringlet

Material examined: 3 km w Mar Saba(PMNH5929, ♂, 13.3.2015); Ain Kenia (PMNH4814, ♂, 15.8.2014; PMNH4830, ♀, 15.8.2014); Auja (PMNH1710-20, ♂♂, 27.3.2014); Salfit (PMNH5166, ♂, 22.8.2013); Mikhmas (PMNH1759-8, ♂♂, 23.5.2013); Wadi Al Qelt (PMNH6205, ♀, 4.4.2015; PMNH6207, ♂, 4.4.2015; PMNH6208, ♀, 4.4.2015); Wadi Al Quff (PMNH3772, ♂, 3.7.2014; PMNH3940, ♂, 4.11.2014; PMNH3953, ♂, 4.11.2014; PMNH4446, ♀, 6.9.2014; PMNH4456, ♂, 6.9.2014; PMNH4457, ♀, 6.9.2014).

Remarks: Specimens were collected from March to November. This species prefers a warm climate, and it has likely migrated from tropical areas to the Eastern Mediterranean region through the Great Rift Valley (John *et al*., 2010).[2] Larvae feed on Poaceae especially *Hyparrhenia hirta* (Benyamini, 2002a).

Family Nymphalidae

Subfamily Limenitidinae

Limenitis reducta schiffermuelleri Higgins, 1933 (Fig. 1-O) The Southern White Admiral

Material examined: Al Makhrour-Bethlehem (PMNH6331, ♀, 15.4.2015); Nabi Saleh (PMNH4798, ♂, 21.7.2014); Ain Kenia (PMNH4803, ♂, 15.8.2014); Salfit (PMNH5081, ♀, 22.8.2014); Al Walaja (PMNH4615, ♂, 8.8.2014).

Remarks: We collected specimens in April, July, and August. Larsen and Nakamura (1983) used subfamily Nymphalinae for this species; however, Wahlberg *et al*. (2003) found that it belongs to the subfamily Limenitidinae. The Southern White Admiral ranges globally from southern and central Europe to Iran. Locally, it is widespread in the well wooded areas like Salfit and Al Makhrour. According to Benyamini (1997) it flies from April till mid-October in northern and central Palestine. *L. reducta* is known to feed on *Lonicera* sp. (Larsen, 1974).

Subfamily Nymphalinae

Melitaea deserticola macromaculata Belter, 1934 Desert Fritillary

Material examined: Mar Saba (PMNH6427, ♂, 15.3.2015; PMNH5962, ♂, 13.3.2015); Wadi Al Quff (PMNH3917, ♀, 21.3.2014; PMNH3918,♂, 21.3.2014; PMNH4078, ♂, 21.4.2014; PMNH4103, ♂, 5.3.2014; PMNH4455, 6.9.2014).

Remarks: We collected specimens in March, April, and September. The genus *Melitaea* belongs to Melitaeini tribe. According to Larsen & Nakamura (1983), this Palaearctic species is mostly found in North Africa and Levant regions. In Palestine, *M. deserticola* is found in northern and middle areas, including the Jordan Valley (Benyamini, 1983). The Desert Fritillary is observed from February to May and in October (Benyamini, 1997), which is confirmed by the PMNH team when they caught it in early September; this leads us to agree with Larsen and Nakamura (1983) in that *M. deserticola* has three broods - the second and third are irregular - with the first being the largest brood. Larvae feed on Scrophulariaceae, especially *Anarrhinum orientalis, Linaria aegyptiaca* and *Kickxia aegyptiaca* (Larsen & Nakamura, 1983; Larsen, 1990).

Melitaea telona Fruhstorfer, 1908 The Knapweed Fritillary

Material examined: Al Makhrour-Bethlehem (PMNH6301, ♂, 15.4.2015), Wadi Al Quff (PMNH4461, 9.6.2014; PMNH4104, ♂, 5.3.2014), Wadi Al Qelt (PMNH6426, ♂, 2.4.2015).

Remarks: We collected specimens in March, April, and June. Tóth & Varga (2010) found that *M. phoebe* is a distinct species and is confined to the Euro-Siberian region, while *Melitaea telona* is Ponto-Mediterranean. In Palestine, it is widely common in northern Negev, being the most southern regions (Benyamini, 1983). Unlike the Jordanian population, our population seems to have only one brood in early spring. Its larvae feed on *Scabiosa, Centaurea,* and *Plantago* (Larsen, 1974).

Melitaea trivia syriaca Rebel, 1905 The Mullein Fritillary

Material examined: Wadi Al Quff (PMNH4458, 9.6.2014).

Remarks: We collected one specimen of this species in June, and according to Benyamini (1997), *M. trivia* was observed from March to early November. This species occurs in the hot parts of southern Europe to central Asia, including the Middle East. It is known in north and middle Palestine and almost to central Negev (Benyamini, 1997, 1983). Its larvae feed on *Verbascum* sp. (Katbeh-Bader *et al*., 2003).

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[2] For nearby area distributions, see: Larsen & Nakamura (1983); Amr *et al*. (1997); Benyamini (2002a, b); Katbeh-Bader *et al*. (2003).

Polygonia egea (Cramer, 1775) The Southern Comma

Material examined: Al Makhrour-Bethlehem (PMNH6544, ♂, 3.5.2015), Bethlehem (PMNH5144, ♂, 28.8.2014; PMNH5334, ♂, 21.6.2014; PMNH5335, ♂, 19.6.2014; PMNH5336, ♂, 12.8.2014).

Remarks: Specimens were collected in May, June and August. The Southern Comma is found along the Mediterranean coast from Provence to Greece, through Turkey and the Levant to Afghanistan (Larsen & Nakamura, 1983). According to Benyamini (1997), *P. egea* was observed from January to August. When *P. egea* closes its wings, it looks like an old and dry leaf. *P. egea* is found along the Mediterranean, Asia Minor to north India; it is also found in north and middle Palestine; the common food plants are species of *Parietaria* (Benyamini, 1997; Larsen, 1974 & 1983).

Vanessa atalanta (Linnaeus, 1758) The Red Admiral

Material examined: Bethlehem (PMNH5666, 30.1.2015; PMNH6358, 23.3.2015; PMNH6363, 15.3.2015; PMNH5262, 12.3.2014); Wadi Al Qelt (PMNH6347, 28.2.2015).

Remarks: Collected in January, February and March. The Holarctic Red Admiral is found in most parts of Palestine, except for the Negev (Benyamini, 1983). Larsen (1974 & 1990) reported that its larvae feed on *Urtica* sp. and *Parietaria* sp., especially *Parietaria alsinifolia.* According to Larsen (1990), *V. atalanta* lay eggs where it cannot survive, a reason for its migration behavior.

Vanessa cardui cardui (Linnaeus, 1758) Painted Lady

Material examined: Ain Hijla (PMNH3995, 18.4.2014); Al Aqaba (PMNH4048, 18.4.2014; PMNH4049, 18.4.2014); Bardala (PMNH4052, 18.4.2014), (PMNH4054, 18.4.2014); Bethlehem (PMNH5665, 30.1.2015; PMNH6353, 24.2.2015; PMNH5313, 3.7.2014; PMNH5965, 13.3.2015; PMNH5337, 16.5.2014); Mar Saba-Bethlehem (PMNH5959, 13.3.2015; PMNH5946, 13.3.2015); Matahen Al Sukkar-Jericho (PMNH5546, 21.1.2015); Wadi Al Qelt (PMNH5765, 23.2.2015); Wadi Al Quff (PMNH3958, 4.11.2014; PMNH4029, 21.4.2014; PMNH4080, 21.4.2014).

Remarks: This is a rather common species and was collected during most of the year. *V.cardui* has a worldwide distribution but it is rare in the tropical areas (Larsen, 1974, 1983 & 1990). According to Benyamini (1997), the Painted Lady is found all over Palestine. It migrates to the north in both Lebanon and Palestine during March and April and it migrates from Jordan to Saudi Arabia. *V. cardui* larvae feed on *Malva parvifolia* and *Malva sylvestris (*Larsen, 1983).

Family Lycaenidae

Subfamily Theclinae

Satyrium spini melantho (Klug, 1832) Blue Spot Hairstreak

Material examined: Nabi Saleh (PMNH1736-25, ♀♀, 3.4.2013).

Remarks: We collected one sample through our trips in April from Nabi Saleh. The Blue Spot Hairstreak is found in South and Central Europe and the Middle East to Iran. *S. spini* is found in north and central Palestine, during late spring and summer (Benyamini, 1997). Its larvae feed on *Rhamnus* spp. (Rhamnaceae) and possibly oak (Larsen, 1974; Halperin & Sauter, 1992).

Subfamily Aphnaeini

Apharitis acamas (Klug, 1834) Arab Leopard Butterfly

Material examined: Wadi Fukeen (PMNH 4690, ♀, 9.8.2014); Al Walaja (PMNH4611, ♀, 8.8.2014).

Remarks: The Leopard Butterfly is an Eremic butterfly with a wide distribution range in the Arab World with different subspecies; two of them, *acamas* Klug and *egyptiaca* Riley, are likely to occur in Palestine. *A. acamas* flies from early spring till late summer and it has three broods according to Benyamini (1997) and Larsen & Nakamura (1983).

Subfamily Lycaeninae

Lycaena phlaeas (Linnaeus, 1761) The Small Copper Butterfly

Material examined: Bethlehem (PMNH6435, ♀, 20.4.2015); Wadi Al Qelt (PMNH6203, ♂, 4.4.2015).

Remarks: Specimens were collected in April. The small copper has a worldwide distribution from the United States of America to Asia and Africa with Europe in the middle. In Palestine, *L. phlaeas* is dominant in agricultural lands, from the most northern areas till Hebron, being in the south, including Jericho, 500m below the sea level (Benyamini, 1983). Like *L. thersamon, L. phlaeas* feeds on *Rumex* sp., *Sarothamnus* sp. and *Polygonum* sp. (Katbeh-Bader *et al.*, 2003).

Lycaena thersamon (Esper, 1784) (Fig. 1-J) Lesser Fiery Copper

Material examined: Ain Kenia (PMNH4807, ♂, 15.8.2014); Al Makhrour – Bethlehem (PMNH6302, 15.4.2015); Al Walaja (PMNH4591, ♀, 19.7.2014); Artas (PMNH4714, 12.8.2014; PMNH4712, ♀, 12.8.2014; PMNH4715, ♀, 12.8.2014; PMNH4735, ♀, 13.8.2014); Bethlehem (PMNH4597, ♂, 22.7.2014; PMNH5331,♂, 15.8.2013; PMNH5332, ♂, 09.8.2013; PMNH6433, ♀, 20.4.2015); Burak Sulaiman (PMNH4951, ♂, 17.8.2014; PMNH4953, ♀, 17.8.2014); Salfit (PMNH5060, ♂, 22.8.2014; PMNH5076, ♂, 22.8.2014; PMNH5080, ♂, 22.8.2014; PMNH5088, ♂, 22.8.2014; PMNH5091, ♂, 22.8.2014; PMNH5152, ♂, 22.8.2014); Wadi Al Quff (PMNH3961, ♂, 4.11.2014; PMNH4453, ♀, 6.9.2014).

Remarks: We collected specimens from April to November. This species has a distribution extending from Italy and Austria to the Balkans; it is more focused in Middle East regions (Larsen, 1990) with a vast distribution in Palestine. Katbeh-Bader *et al.* (2003) suggested that two broods occur: One in April and the other in August and that the larvae live on *Rumex, Sarothamnus* and *Polygonum*. The genus *Lycaena* Fabricius, 1807 has six species in the Levant: *thetis* Klug, 1834, *tityrus* Poda, 1761, *asabinus* Herrich-Schäffer, 1851, *ochimus* Herrich-Schäffer, 1851, *thersamon* Esper, 1784, and *phlaeas* Linnaeus, 1761. Two subspecies (*thersamon* Esper, 1784, *omphale* Klug and *kurdistanica* Riley) were suspected to occur in the Levant; however, we noticed a wide variation among our samples, so we did not assign a specific sub-level to our specimens.

Subfamily Polyommatinae

Aricia agestis agestis (Denis & Schiffermüller, 1775) The Brown Argus

Material examined: Ain Shibly- Bardala (PMNH6552, ♀, 6.5.2015; PMNH6555, ♀, 6.5.2015; PMNH6558, ♀, 6.5.2015); Beit Jala (PMNH5309, ♀,12.8.2014); Bethlehem (PMNH 6413, ♂, 26.3.2015; PMNH5311, ♀, 22.5.2014), Salfit (PMNH5095, ♀, 22.8.2014).

Remarks: Specimens were collected in March, May and August. *A. agestis* is known in Europe, the Levant and Iran. The Brown Argus occurs in central Palestine, and some populations, in the northern areas of Palestine, are connected with the Lebanese populations (Benyamini, 1983). Its larvae feed on *Erodium cicutarium* and *Helianthemum* sp. (Larsen, 1974).

Azanus ubaldus (Stoll, 1782) Desert Babul Blue

Material examined: Ain Hijla (PMNH4013, ♂, 18.4.2014).

Remarks: We collected a single specimen in April. *A. ubaldus* occurs in North Africa, the Middle East and India (Larsen, 1990). In Palestine, it is common in the arid areas near water surface (Benyamini, 1983). Larvae of the Desert Babul Blue feed on *Acacia* sp. and pupate under stones (Larsen, 1990).

Freyeria trochylus **(**Freyer, 1845) (Fig. 1-K) The Grass Jewel

Material examined: Al Walaja (PMNH5249, ♂, 20.9.2014); Beit Ta'mar (PMNH5284, ♂, 22.10.2014; PMNH5340, ♂, 28.6.2014); Salfeet (PMNH5096, ♂, 22.8.2014); Wadi Al Qelt (PMNH5895, ♂, 9.3.2015; PMNH 5894, ♂, 9.3.2015); Wadi Al Quff (PMNH4459, ♂, 9.6.2015).

Remarks: We collected specimens from March to October. *Freyeria trochylus* is widespread in Africa, the Balkans, the Middle East, India, and other oriental regions (Larsen & Larsen, 1980). The Grass Jewel is found throughout Palestine and all year round, except in January (Benyamini, 1997). The larval food plants are *Heliotropium* and *Indigofera* (Larsen & Nakamura, 1983).

Lampides boeticus **(**Linnaeus, 1767) (Fig. 1-L) The Long-tailed Blue

Material examined: Bethlehem (PMNH6981, ♀, 21.6.2015).

Remarks: This is one of the most widespread species occurring in the Palearctic region and in Africa and from England to Japan. Phylogenitically, it likely originated in Africa some 7 million years ago and spread from there (Lohman *et al*., 2008). Halperin and Sauter (1992) reported *Colutea* and *Tephrosia* (Fabaceae) as larval food plants.

Leptotes pirithous (Linnaeus, 1767) Lang's Short-Tail Blue

Material examined: Beit Jala (PMNH5333, ♀, 8.8.2015).

Remarks: A single specimen was collected in August. *Leptotes pirithous* is known in southern Europe and most of Africa and the Middle East. In Palestine, it occurs in all the country all year round (Benyamini, 1997). Larvae feed on leguminous plants (Fabaceae) and *Plumbago* sp. (Plumbaginaceae) (Larsen, 1990; Halperin & Sauter, 1992).

Polyommatus icarus **(**Rottemburg, 1775) Common Blue

Material examined: 3 km w Mar Saba (PMNH5952, ♀, 13.3.2015; PMNH5958, ♂, 13.3.2015); Al Makhrour–Bethlehem (PMNH6308, ♂, 15.4.2015); Al Qarn (PMNH4586, ♀, 7.7.2015); Ain Kenia (PMNH4813, ♀, 15.8.2014; PMNH4819, ♂, 15.8.2014; PMNH4837, ♂, 15.8.2014); Ain Shibly-Bardala (PMNH6556, ♂, 6.5.2015); Artas (PMNH4710, ♂, 12.8.2014; PMNH4716, ♂, 12.8.2014); Bethlehem (PMNH6367, ♂, 26.3.2015;PMNH6414, ♂, 16.3.2015; PMNH6377, ♂, 26.3.2015; PMNH6376, ♀, 27.3.2015; PMNH6370, ♂, 21.3.2015; PMNH5310, ♀, 18.5.2014; PMNH5312, ♀, 30.6.2014; PMNH5316, ♂, 2.7.2014; PMNH4599, ♀, 22.7.2014; PMNH4598, ♂, 22.7.2014); Beit Fajjar (PMNH5116, ♀, 23.8.2014); Husan (PMNH1954-10, ♀♀, 17.6.2013; PMNH1954-7, ♂♂, 17.6.2013); Nabi Saleh (PMNH1736-5, ♂♂, 3-4.5.2013); Salfit (PMNH5071, ♂, 22.8.2014; PMNH5089, ♂, 22.8.2014; PMNH5103, ♂, 22.8.2014); Wadi Al Quff (PMNH3914, ♂, 21.3.2014); Silit Adahr (PMNH18003-5, ♂♂, 14.6.2013); Wadi Fukeen (PMNH5141, ♂, 29.8.2014; PMNH5142, ♂, 29.8.2014);Wadi Al Abyad, 6 km NW of Nuwaima (PMNH6430, ♀, 24.4.2015; PMNH6431, ♀, 24.4.2015); Wadi Al Qelt (PMNH6422, ♂, 2.4.2015; PMNH6421, ♂, 2.4.2015); Zatara (PMNH6084, ♀, 22.4.2014).

Remarks: Specimens were collected in March, April, May, June, July, and August. The genus *Polyommatus* has 183 species and needs further studies at the molecular and morphological level (Talavera *et al*., 2012). Two subspecies of *P. icarus* were previously reported in Palestine: *zelleri* Verity, 1919 and *juno* Hemming, 1933. But we noted significant variations and intergradation in coloration, and we do not suggest using subspecies names here. Furthermore, population genetic studies illustrate that there are few genetic differences between the different populations of *P. icarus* in southern Europe (Schmitt *et al*., 2003).

Tarucus balkanicus **(**Freyer, 1845) Little Tiger Blue

Material examined: Ain Shibly- Bardala (PMNH6557, ♂, 6.5.2015).

Remarks: One specimen of this Eremic species was collected in May from Ain Shibly. *T. balkanicus* is found in the Eremic part of Africa, the Balkans, Asia Minor and the Far East (Larsen, 1974). In Palestine, the Little Tiger Blue is found in the Jordan Valley and most of northern Palestine and in the southern Palestinian Mediterranean coast all year round, except in January and February (Benyamini, 1983). Larvae feed on *Zizyphus vulgaris*, *Z. spina-christi, Paliurus spina-christi* and *P. vulgaris* (Larsen, 1974).

Tarucus rosaceus (Austaut, 1885) (Fig. 1-M) The Mediterranean Pierrot

Material examined: Ain Hijla (PMNH3996, ♀, 18.4.2014); Jericho (PMNH5880, ♂, 9.3.2015; PMNH5263, ♂, 9.8.2013).

Remarks: Collected in March, April, and August. *T. rosaceus* has a wide distribution extending from North Africa and Middle East to India. In Palestine, it is more common on the border line with Jordan, including the Jordan Valley and Jericho all year round (Benyamini, 1997). Its larvae feed on *Zizyphus* and *Paliurus* (Rhamnaceae) (Larsen, 1990).

Zizeeria karsandra (Moore, 1865) The Asian Grass Blue

Material examined: Wadi Al Abyad, 6 km NW of Nuwaima (PMNH6429, ♀, 24.4.2015).

Remarks: This is the smallest butterfly collected in this survey. It is hard to find because of its small size, so it may be more common than thought. The Asian Grass Blue found from Australasia, via India, to Oman, Iraq, Lebanon, Egypt, Libya and Tunisia, and it is common in all Palestine, except in west Negev all year round (Benyamini, 1997). Larvae feed on *Trifolium alexandrinum* and other related plants (Larsen, 1974).

Family Hesperiidae

Subfamily Pyrginae

Carcharodus alceae alceae (Esper, 1780) The Hollyhock Skipper

Material examined: Ain Kenia (PMNH4799, ♂, 15.8.2014); Beni Neim (PMNH5711, ♀, 17.2.2015); Bethlehem (PMNH5314, ♀, 8.8.2013; PMNH5315, ♀, 3.7.2014), Edna (PMNH4998, ♀, 23.8.2014); Wadi Al Qelt (PMNH6425, 27.3.2015).

Remarks: Specimens were collected in July and August. This species has a wide-range of distribution extending from Europe to the Mediterranean area. It has three broods (Benyamini, 1997; Larsen and Nakamura, 1983). The larvae feed on species of the family Malvaceae such as *Althaea, Malva*, and *Alcea* (Alexiou, 2014; Benyamini, 1984, 1997; Gilbert & Zalat, 2007; Larsen, 1990; Larsen & Nakamura, 1983).

Pyrgus melotis **(**Duponchel, 1834) The Levantine Grizzled Skipper

Material examined: Salfit (PMNH5104, ♀, 22.8.2014).

Remarks: We collected one specimen in August of this Palearctic species. Palestine is the most southern range of its distribution and represented by the subspecies *P. melotis melotis* (DeJong, 1987). It prefers moist habitats, such as small permanent springs bordered by *Rubus* (Larsen & Nakamura, 1983). Apparently, one brood is formed in the spring, while, in Lebanon, Larsen (1974) indicated that two generations appear.

Spialia orbifer hilaris (Staudinger, 1901) The Orbiferous Skipper

Material examined: Ain Kenia (PMNH4831, ♀, 15.8.2014); Al Makhrour-Bethlehem (PMNH6039, 15.4.2015; PMNH6337, 15.4.2015; PMNH6303, 15.4.2015; PMNH6209, 15.4.2015); Al Walaja (PMNH4632, ♂, 8.8.2014); Wadi Al Abyad (PMNH6501, ♀, 24.4.2015); Wadi Al Quff (PMNH4031, ♀,21.4.2014).

Remarks: We collected specimens in the months of April and August. The Orbiferous Skipper occurs in a series of subspecies in Yugoslavia, the Middle East, Russia, western China and Korea. In Palestine, it is confined to the Mediterranean zone. Larsen & Nakamura (1983) stated that two broods are produced, one in early April and the second in July.

Syrichtus proto hieromax Hemming, 1932 The Large Grizzled Skipper

Material examined: Edna (PMNH5003, ♀, 23.8.2014).

Remarks: A single specimens was collected in August. Large Grizzled Skipper is a Mediterranean butterfly found in North Africa, Iberian Peninsula, Turkey and the Levant (Larsen & Nakamura, 1983). The subspecies *hieromax* was originally described in Ajlune [Ajloun], Jordan (Hemming, 1932), and seems to be localized in Jordan, Palestine and the coastal region of Lebanon. Larsen & Nakamura (1983) discussed the status of the two subspecies of this form; *Syrichtus proto hieromax* is found in the coastal areas of Lebanon and it is rare in both Jordan and Palestine, and *Syrichtus proto lycaonius* is distributed in the Lebanese mountains. Its larvae feed on *Phlomis* (Lamiaceae) (Larsen & Nakamura, 1983; Halperin & Saute, 1992).

Syrichtus tessellum nomas **(**Lederer, 1855) (Fig. 1-N) The Tessellated Skipper

Material examined: Al Makhrour-Bethlehem (PMNH6339, ♀, 15.4.2015; PMNH6336, ♀, 15.4.2015).

Remarks: Specimens were collected in April. The Tessellated Skipper can be found in the Balkans via the Middle East to Central Asia. The subspecies *nomas* is rare in Jordan (Katbeh-Bader *et al*., 2003). It is quite common in Palestine and Lebanon (Larsen & Nakamura, 1983). Plants like *Phlomis* (Lamiaceae) are larval food plants for this species (Benyamini, 1990; Halperin & Sauter, 1992).

Subfamily Hesperiinae

Gegenes gambica (Mabille, 1878) The Pigmy Skipper

Material examined: Salfit (PMNH5058, ♂, 22.8.2014).

Remarks: A single specimen was collected from Salfit in August. According to Benyamini (1997), *G. gambica* is found from March to December. The Pigmy Skipper is a Mediterranean butterfly. It feeds on grasses (Katbeh-Bader *et al*., 2003).

Thymelicus hyrax hyrax **(**Lederer, 1861)Levantine Skipper

Material examined: Artas (PMNH1711-19, ♀♀, 31.4.2013); Ain Samiya (PMNH1731-12, ♂♂, 12.4.2013); Bethlehem (PMNH6364, ♀, 26.3.2015; PMNH6368, ♂, 26.3.2015; PMNH6411, ♂, 16.3.2015).

Remarks: We collected specimens in March and April. The Levantine Skipper is common in the Levant from Turkey east to Iran and south to Palestine. In Palestine, it is found in the central and northern areas from April till early July (Benyamini, 1997). The Levantine Skipper feeds on *Dianthus multipunctatus* (Larsen, 1974).

Thymelicus sylvestris syriaca (Tutt, 1905) Small Skipper

Material examined: Bethlehem (PMNH6361, ♂, 27.3.2015; PMNH6368, ♂, 26.3.2015; PMNH6374, ♂, 21.3.2015; PMNH6373, ♂, 21.3.2015; PMNH6371, ♀, 21.3.2015; PMNH6385, ♂, 16.3.2015; PMNH6350, ♂, 28.2.2015; PMNH6366, ♂, 26.3.2015; PMNH6412, ♀, 16.3.2015; PMNH5712, 12.2.2015); Mar Saba (PMNH5964, ♂, 13.3.2015; PMNH5954, ♀, 13.3.2015; PMNH5943, ♂, 13.3.2015; PMNH5956, ♂, 13.3.2015; PMNH5934, ♂, 13.3.2015; PMNH5919, 13.3.2015; PMNH5913, ♂, 13.3.2015).

Remarks: We collected the Small Skipper in February and March, mainly in the area of Bethlehem to Mar Saba (an area of 20 km x 20 km). *T. sylvestris* is found in the Mediterranean zone of North Africa, Europe and across Asia Minor to Central Asia and Iran. In Palestine, it is common in the center and the areas around Tiberias (Benyamini, 1997). According to Benyamini (1997), this skipper flies from April to early July, but we collected the specimens in February and March, which means that the brood flies earlier here. Its larval food plants are *Phleum pratense, Holcus mollis* and *Dactylis glomeratus* according to Larsen (1974) who records this species under the name *Adopoea flava syriaca.*

4. Discussion

Palestinian areas have a very rich fauna, including butterflies. Even with this preliminary study, we have managed to record 54 species of butterflies, representing 5 families in a very small part of historic Palestine (parts of the Israeli occupied West Bank). Benyamini (1997) listed 139 species, one third of which was recorded from Sinai (Egyptian territory) and the Golan (occupied Syrian territories) that are not part of historic Palestine. Thus, our sampling has been satisfactory for this initial study of the occupied West Bank. The three most common butterflies observed and collected were the small White *Pieris rapae***,** the Bath White *Pontia daplidice*, and the Common Blue *Polyommatus icarus*. Possible threatened species, based on our preliminary studies are: *Archon apollinus*, *Zegris eupheme*, *Gonepteryx cleopatra taurica*, and *Hipparchia fatua sichaea.*

There have been significant environmental changes that impacted the biodiversity of this area. For example, Qumsiyeh *et al*. (2014) showed a decline in vertebrate biodiversity in the Bethlehem Region. Salman *et al*. (2014) discussed the negative impact of human activities on the amphibian distributions in our areas. For butterflies, we anticipate that a significant decline also occurred and certainly some European butterflies are on the IUCN threatened or near-threatened lists (Van Swaay *et al*., 2011). In our region and from personal observations, we especially highlight the rarity and the potential threat to the species that we saw and were able to collect one or two specimens of each (see above). For example, Sana Atallah observed that the orange tufted butterfly *Anthocharis cardamines* was very common in Bethlehem area in the 1960s (from his field notes and collected specimens). However, we were able to see this species only in one area that was fenced in the 1960s and likely protected the local plants that this species feeds on from excessive grazing by livestock. It is also possible that the desertification is driving this and other Mediterranean species towards the more mountainous and northern regions. With the climate change expected to raise temperatures in the next two decades by 2-4 degrees centigrade and decrease the annual rainfall by 20-30% in our region (see Sowers *et al*., 2011), the problems encountered in the decrease in biodiversity might get exacerbated. In addition, the use of insecticides and herbicides on a large scale would certainly affect the populations of the sensitive and rare species.

Our findings of the significant biodiversity in butterflies in the present study are also an underestimation of the total butterfly diversity. Opportunistic sampling, even if carried out in different seasons, usually misses many species (see, for example, Dennis *et al*., 2010). Thus, much more work has to be undertaken on the butterflies of Palestine, including the northern parts of the West Bank and in the Jordan Valley because the current studies have focused mostly on the southern areas (Jerusalem to Hebron). Yet, the present preliminary work highlights the biodiversity in this very small region undergoing dramatic changes in population and habitats that could threaten this important faunistic element.

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Pathogenicity of the Entomopathogenic Fungi *Beauveria bassiana* (Balsamo) and *Verticillium lecanii* (Zimmerman) Against Aphid *Macrosiphum rosae,* Linnaeus (Hemiptera: Aphididae) under Laboratory Conditions

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Abstract

The effects of two entomopathogenic fungi, *Beauveria bassiana* and *Verticillium lecanii* were studied on aphid adults of *Macrosiphum rosae* under laboratory condition (25 ± 1 ºC, 80 ± 5% RH and a photoperiod of 12 L: 12 D h). Bioassay was conducted by putting 1 µl from each concentration on insect body by micro-applicator as topical application method. This experiment was conducted with six replications on CRD design. Laboratory bioassay studies were carried with six different concentrations (10^4, 10^5, 10^6, 10^7, 10^8 spore/ml) of *B. bassiana* and *L. lecanii* on adult aphids of *M. rosae*. The LC_{50} values *L. lecanii* and *B. bassiana* were obtained 1.38×10^4 and 2.66×10^5 spores/ml, respectively. The LT_{50} values ranged from 1.80 to 3.05 days with *L. lecanii* concentrations and from 2.30 to 3.16 with *B. bassiana* concentrations on adult aphids. Results showed that LC_{50} and LT_{50} values *L. lecanii* were lower than *B. bassiana*. Therefore, *L. lecanii* has higher virulence compared with *B. bassiana* on adult aphids of *M. rosae*.

Keywords: *Macrosiphum rosae, Beauveria bassiana, Verticillium lecanii*, LC_{50}, LT_{50}.

1. Introduction

The rose aphid, *Macrosiphum rosae* (L.), is an important pest on rose plants. This pest caused the deformation of the leaf blades, the shortening of shoots, petioles and deformation of the flowers (Salem and Abdel-Raheem, 2015). In addition, this aphid secretes honey-dew and was caused saprophytic fungi developing on plants (Cichocka, 1980; Jaskiewicz, 2006). Aphids may persist throughout the year as colonies on roses (especially in mild winters) and sometimes over winter as eggs on roses (Blackman and Eastop, 2006).

M. rosae has been controlled predominantly by using chemical insecticides, such as primicarb, imidacloprid, parathion, malation, but this method has caused problems for the environment (Talebi Jahromi, 2011). In addition, some studies indicate that chemical insecticides caused resistance in pest populations (Foster *et al.*, 1998).

The biological control with entomopathogenic fungi is gaining importance in pest management programs. In addition, among the different biological agents, entomopathogenic fungi have several advantages compared with the conventional insecticides. Entomopathogenic fungi are, for example, inexpensive, easy for application, high efficiency, non-hazardous for human and ecosystem (Lacey *et al.*, 2001). Therefore, these advantages have led to the commercialization of a large number of new fungus-based biopesticide products (Faria and Wraight, 2007)

Lecanicillium lecanii (Zimmerman) and *Beauveria bassiana* (Balsamo) have been recognized as entomopathogenic fungi with high potential in biological control of aphids (Askary *et al.*, 1998; Derakhshan *et al.*, 2007; Abd El-Salam and El-Hawary, 2011). The purpose of this research is to evaluate the bio-efficacy of *Verticillium lecanii* and *B. bassiana* on adult aphids of *M. rosae* under laboratory condition

2. Materials and Methods

2.1. Insect Rearing

Rose aphids were collected from the rose plants of the University of Mohaghegh Ardabili, Ardabil, Iran. Thereafter, aphids were transferred to rose plants of Floribunda cultivar. All the treated Petri dishes were

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maintained at 25 ± 1 °C, 80 ± 5 % RH and a photoperiod of 12: 12 h (L: D) in an incubator.

2.2. Entomopathogenic Fungi

Commercial formulation enthomopathogenic fungi of *B. bassiana* and *V. lecanii* were obtained from Sadrabiotech Company of Iran.

2.3. Bioassay

Five different concentrations (1×10^8, 1×10^7, 1×10^6, 1×10^5, 1×10^4 spores/ml) were prepared for *B. bassiana* and *V. lecanii.* Conidial suspensions were vortexes for 5 min to produce a homogeneous suspension. Each concentration was replicated six times and each replication consisted of 10 adult aphids. Pathogenic fungi bioassay was conducted by putting 1µl from each concentration on insect body by microapplicator as topical application method. One day old adult aphids were transferred using a camel hairbrush on broad rose leaves into Petri dishes (11 cm diameter) that were filled with thick layer of 0.1% agar. Totally, 60 aphids were used for each treatment. For controls, adult aphids were treated with distilled water. The mortality data were recorded over a period of five days and rose fresh leaves were used each day for feeding aphids.

2.4. Statistical Analysis

The LC_{50} and LT_{50} values were calculated using the Probit procedures with SPSS for Windows® release 19.0. The percent corrected cumulative mortality of each fungus was subjected to ANOVA test and the means were compared by the Tukey test, using SPSS 19.0 software program (SPSS, 2009). For the correction mortality data with that in control used the Abbott's formula (Abbott, 1925).

3. Results and Discussion

The data presented in Table 1 shows the LC_{50} values of *V. lecanii* and *B. bassiana* at 3th day post treatment on *M. rosae*. According to this table, the LC_{50} value of *V. lecanii* is lower than *B. bassiana* on aphids (Figures 1 and 2). Therefore, *V. lecanii* is more pathogenic compared with *B. bassiana* on adult aphids *M. rosae*. Abd El-salam and El-Hawary (2011) reported that LC_{50} *L. lecanii* is lower than *B. bassiana* on *Aphis craccivora* (Koch). In addition, Sarnyaya *et al.* (2010) indicated that *V. lecanii* was more pathogenic than *B. bassiana* against adults *Brevicoryne brassica* (Linnaeus). Therefore, the similarity between the present and the above-mentioned studies indicates that *L. lecanii* is more pathogenic compared with *B. bassiana* on aphids. Abdel-Raheem *et al.* (2015) studied the efficacy of *B. bassiana* and *V. lecanii* against three life phases of *Tuta absoluta*. Results indicated that LC_{50} *B. bassiana* is lower compared to *V. lecanii* on larvae (Neonate- 2nd and 3rd instare). Therefore, *B. bassiana* is more effective than *V. lecanii* on *T. absoluata*, which disagrees with our findings. Therefore, this difference might be due to the difference in the virulence of fungal isolates and the host species.

LC_{50} values of *V. lecanii* and *B. bassiana* were obtained: 1.38×10^4 and 2.66×10^5 spore.ml^{-1}, respectively. Nazemi *et al.* (2014) found that LC_{50} of *Lecanicillium longisporum* on the aphid *Cinara pini* (Linnaeus) was 1.2×10^6 spore.ml^{-1}. Vu *et al.* (2007) reported that the LC_{50} value of *L. lecanii* against *Myzus persicae* (Sulzer) was 1.65×10^6 spore.ml^{-1} after six days of the treatment. In addition, Akmal *et al.* (2013) indicated that LC_{50} *B. bassian* on *B. brassica* and *Schizaphis graminum* (Rondani) were 6.28×10^5 and 6.76×10^6 spore.ml^{-1}, respectively. Comparing the above-mentioned studies with the present study indicated that LC_{50} *L. lecanii* and *B. bassiana* on *M. rosae* are lower

Lethal time values for 50% mortality (LT_{50}) ranged from 1.80 to 3.05 days with *L. lecanii* concentrations and from 2.30 to 3.16 days with *B. bassiana* concentrations on adult aphids *M. rosae* (Table 2). Results indicated that increasing concentration caused reducing the LT_{50} and the lowest LT_{50} were obtained at the highest concentration (10^8spore/ml). The LT_{50} values of *L. lecanii* in all concentration were significantly lower compared with *B. bassiana* on adult aphids *M. rosae*. Sarnyaya *et al.* (2010) reported that the LT_{50} values of *L. lecanii* and *B. bassiana* on *A. carcivora* were 3.90 to 7.25 and 3.63 to 6.88 days, respectively. Therefore, their findings disagree with the results of the present research.

Also, the present study indicated that mortality adult aphids increased with increasing the conidial concentrations and the time placed after treatment. A 100% mortality was obtained in concentrations 10^7 and 10^8 spore/ml with *L. lecanii* on the 4th day (Table 4). But *B. bassiana* caused 100% mortality by these concentrations on the 5th day post treatment (Table 3). Loureiro and Moino (2006) found that *B. bassiana* caused 100% mortality on *M. persicae* at 10^6 spore's ml^{-1} on the 7th day of treatment. Abd El-salam and El-Hawary (2011) recorded 100 % mortality of adults *A. carcivora* with *L. lecanii* applied at concentration 1.0 ml conidial suspension (1×10^6 spore/ml) after three days. So, Sarnya *et al.* (2010) reported that *L. lecanii* caused 100% mortality at 10^7 and 10^8 spore.ml^{-1}, but *B. bassiana* caused mortality 96% at 10^8 spore.ml^{-1} after 7 days. Results of the studies above are different from our findings; therefore, this difference might be due to the difference in the virulence of fungal isolates and the host species.

This leads to the conclusion that LC_{50} and LT_{50} of *V. lecanii* against *M. rosae* are lower compared with *B. bassiana*. Therefore, the present study suggests that the entomopathogenic, *V. lecanii* is more suitable for control *M.rosae* compared to *B. bassiana.*

Table 1. LC_{50} values of *V. lecanii* and *B. bassiana* against adult aphides, *M. rosae*.

Fungi	N	LC_{50} (spore/ml)	95% Confidence lower – upper	Slope ±SE	X^2
Verticillium lecanii	360	1.38×10^4	8.82×10^2-6×10^4	0.29±0.05	4.30
Beauveria bassiana	360	2.66×10^5	8.59- 6.79×10^6	0.11±0.06	0.62

Table 2. LT_{50} values of *V. lecanii* and *B. bassiana* against adult aphides, *M. rosae.*

Fungi	Concentration(spore/ml)				
	10^8	10^7	10^6	10^5	10^4
Verticillium lecanii	1.80	2.12	2.52	2.83	3.05
Beauveria bassiana	2.30	2.60	2.84	3.07	3.16

Table 3. Mortality percentage of *M. rosae* treated with *B. bassiana*

Day Concentration (Spore/ml)	1	2	3	4	5
Control	0	0	0	0	0
10^4	0	16.66	45	71.66	100
10^5	0	20	48.33	71.66	100
10^6	0	26.66	50	78.33	100
10^7	15	31.66	55	86.66	100
10^8	18.33	38.33	65	93.33	100

Table 4. Mortality percentage of *M. rosae* treated with *V. lecanii.*

Day Concentration (Spore/ml)	1	2	3	4	5
Control	0	0	0	0	0
10^4	0	18.33	51.66	75	100
10^5	0	23.33	60	80	100
10^6	0	36.66	65	90	100
10^7	15	45	75	100	100
10^8	20	53	93.33	100	100

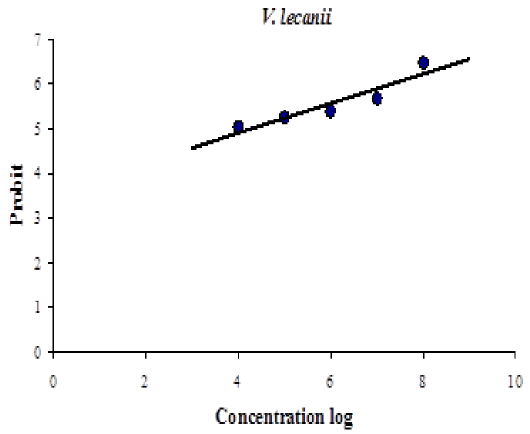

Figure 1. Probit graph for *V. lecanii* on 3 day

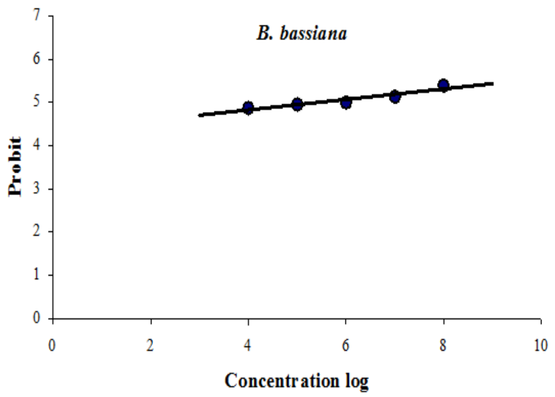

Figure 2. Probit graph for *B. bassiana* on 3 day

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Macroscopic and Microscopic Findings in *Theileria lestoquardi* Naturally Infecting Sudanese Sheep

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Abstract

Malignant Ovine Theileriosis (MOT), caused by *Theileria lestoquardi*, is a major constraint for sheep production in many areas of the world including Sudan. Export sheep and sheep products are a major component of Sudan national economy and foreign income. Despite the importance of the disease, there is a considerable lack of detailed information regarding the postmortem and histological manifestations in the infected sheep. To specify the macroscopic and microscopic findings in sheep infected with MOT, 45 Sudanese sheep, *T. lestoquardi* negative were selected and were maintained for 3 months under natural ticks challenge. Necropsy was performed on 15 dead infected and 4 euthanized non-infected control, all pathological lesions were recorded. Kidneys, liver, lungs, spleen, heart, lymph nodes, stomach, intestine, pancreas and brain from test and control groups were sampled, fixed and were processed for histopathological examinations. The 15 infected sheep displayed severe enteritis with scattered areas of petechial hemorrhages on the serosal and mucosal surface along the small and large intestines. In most animals (n = 7-14), their superficial lymph nodes, liver and spleen were enlarged and their gall bladder were distended. Heart showed petechial hemorrhages and kidneys were congested. All infected animals (100%) revealed sever pneumonia associated with edema and frothy exudates. Comparatively, the most remarkable microscopic lesions in infected sheep were obviously seen in the lungs which exhibited emphysema, congestion, collapse and proliferation of large mononuclear cells. The present study indicates that *T. lestoquardi* infections are accompanied by severe pulmonary involvements, suggesting that emphysema and interstitial pneumonia may lead to respiratory failure and could provide evidence for death. Our findings may assist our knowledge about the microscopic and macroscopic lesions caused by *T. lestoquardi* and could contribute to raise awareness among veterinary authorities regarding the pathognomonic lesions for early and/or differential diagnosis.

Keywords: Sheep, Sudan, Theileria lestoquardi, macroscopic and microscopic lesions, Pneumonia.

1. Introduction

Theileria lestoquardi (Morel and Uilenberg, 1981) is a tick-borne protozoan parasite of sheep, transmitted by *Hyalomma anatolicum* (Tageldin *et al*., 2005; Taha and El Hussein, 2010) and causes a disease known as Malignant Ovine Theileriosis (MOT). The disease was first described in Egypt in exported Sudanese sheep and was subsequently reported in Sudan (Tageldin *et al*., 1992; Latif *et al*., 1994; El Ghali *et al*., 1994), Saudi Arabia (El-Metenawy, 1999; El-Azazy *et al*., 2001) and in Sultanate of Oman (Tageldin *et al*., 2005). Sudan is endowed with large livestock wealth, 36% of which is sheep (Sulieman *et al*., 1990) and it is of particular importance (Abualazayium, 2004). Accordingly, export sheep and their products are a major component of the national economy and foreign income. So far, the disease prevalent in different parts of Sudan, where up to 23% sero-prevalence rate (Salih *et al*., 2003) and 100% mortality in outbreaks were reported (Latif *et al*., 1994; El Ghali and El Hussein, 1995). Therefore, the improvement of the sheep production in the country is hampered.

Sheep are considered as very receptive host for natural *T. lestoquardi* infection that evolves as sub-acute and acute theileriosis (Tageldin *et al*., 1992, 2005, El Hussein *et al*., 1998, El Imam *et al*., 2015). The objective of the present study was to specify the macroscopic and microscopic findings in sheep infected with MOT under natural conditions.

2. Materials and Methods

The experiment was designed to study the macroscopic and microscopic alterations of naturally infected sheep with *T. lestoquardi* and was conducted

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according to the animal ethics guidelines which guarantee that animals do not unnecessarily suffer. A total of 45 apparently healthy male sheep, 4 to 5 months old, were purchased from known disease free districts (Hassan and Salih, 2009); they were introduced to the known *T. lestoquardi* endemic focus in Atbara, Northern Sudan (Taha *et al*., 2013) and maintained for 3 months under natural ticks challenge. Maintenance of the experimental sheep and the clinical observations are described elsewhere (El Imam *et al*., 2015). Treatment was initiated for recumbent or progressively emaciated animals using buparvaquone at a dose of 2.5 mg/kg body weight and *in the extremis* some animals were euthanized using barbiturate (Sodium pentobarbital)100 mg/kg IV, (D Special, Shering-Plough Animal Health, Germany). The animals were considered infected (n = 15) if they were PCR-positive for *T. lestoquardi* and showed schizonts in lymph node biopsy and/or piroplasms in peripheral blood smear and 4 out of 11 PCR-negative sheep were subsequently used as a non-infected control group (n = 4).

2.1. Macroscopic Findings

Within 30 minutes after death, necropsy was performed on dead and/or euthanized sheep. All pathological lesions were recorded in *T. lestoquardi* naturally infected and non-infected control animals.

2.2. Microscopic Findings

For histopathology, 200 tissues specimens (kidneys, liver, lungs, spleen, heart, lymph nodes, stomach, intestine, pancreas and brain) from infected and control animals were sampled, fixed and processed using standard methods (Bncroft *et al*., 1996).

2.3. Confirmatory Test for T. lestoquardi Infection

The materials and methods for blood sample, DNA extraction and reaction conditions for conventional PCR to confirm the disease infection were also described elsewhere (El Imam *et al*., 2015).

3. Results

T. lestoquardi schizonts and piroplasms (6.3-14.6/10^2 cells parasitaemia) were detected in 28 animals (6 suddenly died prior showing any significant clinical signs, 7 recovered and 15 were sampled) while the other 17 proved negative.

3.1. Macroscopic Findings

At necropsy, the 15 infected dead sheep displayed severe enteritis of all intestine and congestion of the digestive system with scattered areas of petechial hemorrhages on the serosal and mucosal surface of small and large intestine. Icterus (n = 12) was evident by the diffuse yellowish discoloration of the body fat and fluids (Plate 1). The superficial and mesenteric lymph nodes (n = 14) were variably enlarged (congestion, hemorrhage and/or edema) (Plate 2). The livers (n = 10) were relatively enlarged, congested and showed evidence of fatty change (n = 4) and the gall bladders (n = 9) were distended with viscid greenish bile (Plate 3). The spleens (n = 11) were congested and extremely enlarged with prominent splenic pulp (Plate 4) and contained scattered foci of capsular hemorrhage. The hearts (n = 10) were flabby and showed a petechial hemorrhage in both edocardial and epicardial surfaces. The kidneys (n = 7) were severely congested and the fat around the kidneys was relatively depleted and gelatinous. In addition, the lymph nodes near the hilus were markedly enlarged.

Comparatively, the most prominent and remarkable macroscopic lesions during the different courses of the disease were obviously seen in the lungs. All the infected examined animals (n = 15) revealed severe pneumonia associated with edema and accumulations of creamy-grayish frothy exudate. In 8 infected animals, the lungs lobules were non-collapsed with rubbery texture (interstitial pneumonia) and multiple hemorrhagic foci were diffusely scattered. In addition, the pulmonary lymph nodes (n = 14) were markedly enlarged and edematous.

Plat1 . Photograph of heart showing yellow fat (arrow), indicating jaundice in *T. lestoquardi* infected sheep.

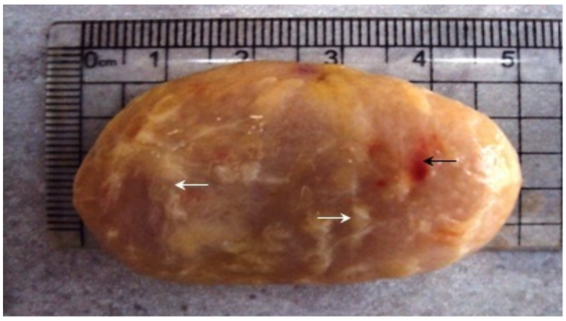

Plate2. Photograph of prescabuler lymph node showing remarkable nodular masses (white arrows) and hemorrhagic foci (black arrow) in *T. lestoquardi* infected sheep.

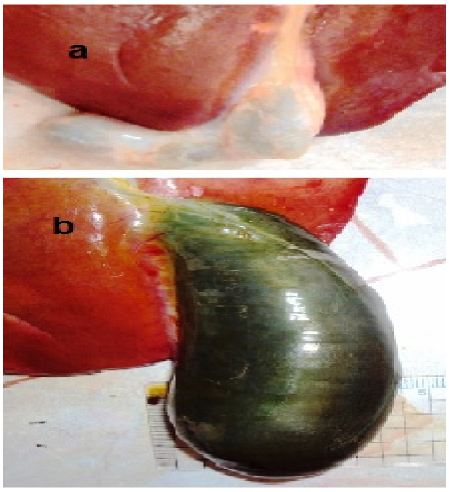

Plate3. Photograph of gall bladder in non-infected control (a) and *T. lestoquardi* infected sheep (b).

Plate4. Photograph of spleen showing splenomegaly and prominent splenic pulps in T. lestoquardi infected sheep.

3.2. Microscopic Findings

The most important histological alterations in *T. lestoquardi* infected sheep were also seen in the lungs and exhibited emphysema, congestion and collapse (Plate 5). Alveolar wall appeared thickened and pneumocytes looked cuboidal with distinct nuclei and infiltrated with round giant cells. In only one section, suppurative bronchopneumonia was diagnosed.

The spleen section showed lymphoid hyperplasia with a prominent white pulp and a periarterial lymphocytic sheath, hemosiderin deposition and mononuclear cells proliferations (Plate 6).

The microscopic lesions of the lymph nodes in many sections (n = 14) showed lymphoid hyperplasia (Plate 7). Lymphoid follicles were distinct but sometimes appeared with proliferating lymphocytes. Medullary sinuses contained large lymphocytes, and the macrophages and the medullary cord were thickened.

In some sections from the heart (n = 6), the muscle cells were widely separated, or closely packed with each other. In few sections, a focal proliferation of interstitial cells was seen with the presence of prominently large mononuclear cells.

The liver in some sections (n = 3) exhibited a marked sinusoidal congestion with dilated central veins and infiltration of portal trials with mononuclear cells. In the congested sinusoid, large mononuclear cells were seen. Many sections (n = 8) showed widened sinusoid, thickened hepatic cord and large monocyte cells in sinusoid. Some of these cells appeared to have more than one nucleus or appear to show cytoplasmic granules. In two cases, capsule was markedly thickened and other two showed distinct cytoplasmic vaculations indicative of fatty change (Plate 8).

Most sections (n = 1 0) of the kidneys appeared normal, though, in all glomerular tuft appears cellular. In few cases (n = 3), the glomerular tuft was either highly cellular or with lobulated tuft or shrunken tuft and widened Bowman's capsule. In two cases, there were few focal areas of interstitial mononuclear cells infiltration. In a number of sections, the tubular epithelial cells, particularly in medulla, sloughed into the lumen.

The sections of the stomach appeared normal but a mucosal edema in four cases and in some mononuclear cells infiltration at the base of gastric glands or in submucosa was seen.

The section of the pancreas, rumen and the intestine appeared normal, but hypercellular of lamina propria was commonly observed in the small intestine.

Vacuolations with gliosis and satillitiosis were the prominent histological changes noticed in the brain sections. In addition only one small area of hemorrhage was seen. No macroscopic and microscopic changes seen in non-infected control animals.

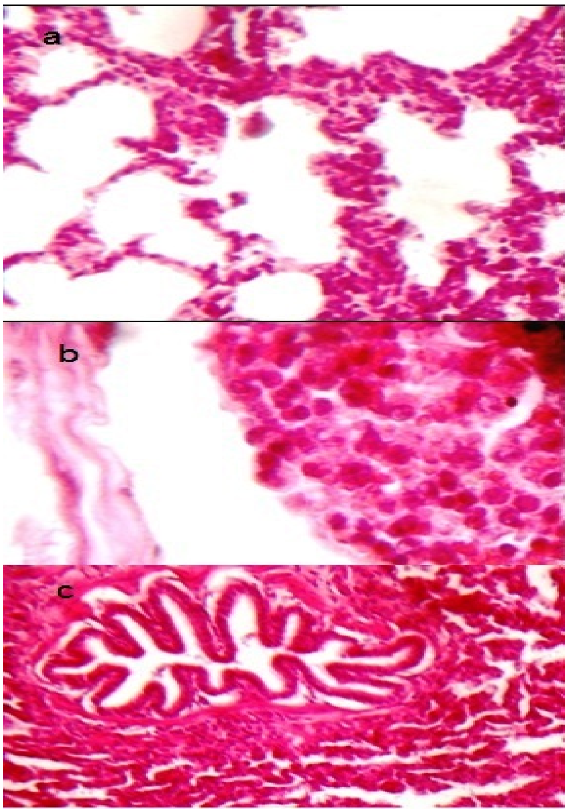

Plate5. Photomicrograph of lung showing (a) emphysema, (b) congestion and (c) collapse in *T. lestoquardi* infected sheep (H&E stain X100).

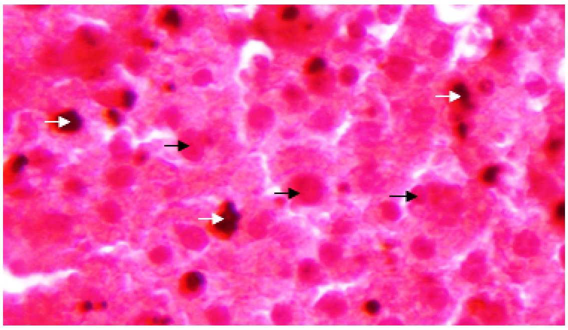

Plate6. Photomicrograph of spleen showing haemosiderin deposit (white arrows) and macrophages (black arrows) in T. lestoquardi infected sheep (H&E stain 100).

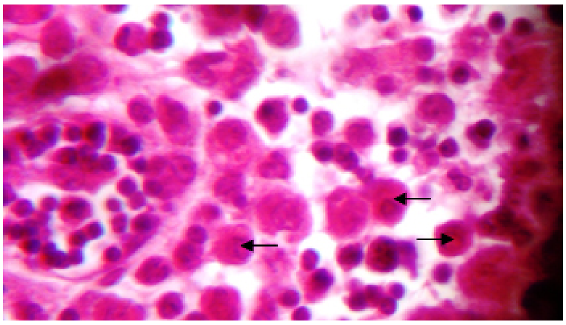

Plate7. Photomicrograph of lymph node showing hyperplasia and large mononuclear cells (arrows) in the medullary sinuses in *T. lestoquardi* infected sheep (H&E stain X100).

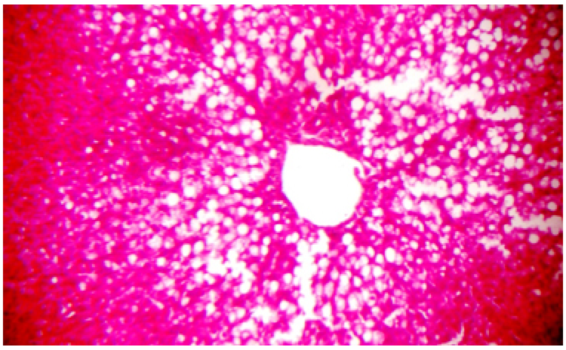

Plate 8. Photomicrograph of liver showing cytoplasmic vaculations indicative for fatty change in *T. lestoquardi* infected sheep (H&E stain X100).

3.3. The Confirmatory Tests for T. lestoquardi Infection

The PCR documentations of *T. lestoquardi* infections are shown in (Figure 1). The PCR confirmatory tests proved all the infected (n = 28) and the non-infected (n = 17) animals were positive or negative for the infection with *T. lestoquardi*.

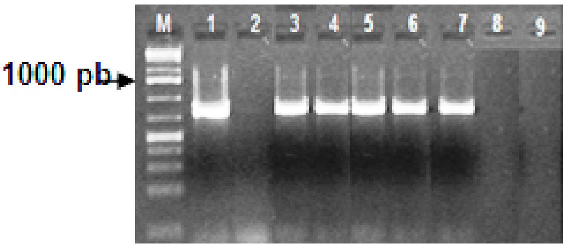

Figure 1. PCR confirmation of *T. lestoquardi* infected and control sheep.Lane M, standard size marker, L_1 positive control, L_2 negative control, L $_{3\text{-}9}$ test samples.

4. Discussion

In Sudan, up to 100% sheep losses during *T. lestoquardi* outbreak were reported (Tageldin *et al.*, 1992). Therefore, *T. lestoquardi* is a lethal disease that causes high morbidity and mortality among naive sheep population if they are exposed to *T. lestoquardi* infected ticks in endemic areas such as Northern Sudan. However, this location is currently considered a suitable region for raising sheep for commercial and export purposes to the neighboring countries.

The pronounced pathology and high mortality are likely to be linked to the ability of *T. lestoquardi* schizonts to stimulate uncontrolled proliferation of the infected leukocyte inducing a phenotype typical of tumor cells (von Schubert *et al*., 2010). The severe enteritis and congestion of the digestive system noticed in the present study could be explained by the fact that sheep are important receptive host for *T. lestoquardi*, as infection usually evolves into sub-acute and acute theileriosis (Tageldin *et al*., 1992;El Hussein *et al*., 1998; Tageldin *et al*., 2005; El Imam *et al*., 2015). The remarkable distension of gall bladder with green bile may be attributed to the heavy destruction of infected RBCs. *T. lestoquardi* infected sheep manifested severe erythrocytes destructions (El Imam *et al*., 2015). Many studies tried to clarify these mechanisms (Shiono *et al*., 2004), where, as observed, morphological changes occur in RBCs surface and increase in its osmotic fragility (Yagi*et al*., 1989), changes in membrane glycolipid components (Watarai *et al*., 1995), oxidative injuries (Shiono *et al*., 2001, 2003; Yagi *et al*., 2002), binding of IgG (Shiono *et al*., 2004) and cytokine tumor necrosis factor (Ahmed, 2002) may play a role in severe RBCs destruction and later result in destructive jaundice.

The disease severity and their pathological changes were similar to the previous reports in Sudan (Tageldin *et al*., 1992; Osman, 1999). The hepatization and the rubbery texture of the infected lungs, observed in addition to the accumulations of excessive fluid and exudate in the chest cavity, were previously reported (Uilenberg, 1981; Irvin and Morrison, 1987; Tageldin *et al*., 2005). Comparatively, lungs exhibited the most prominent microscopic findings of examined organs and proliferation of large mononuclear cells. Macrophages are tissue cells that derive from circulating blood monocytes. Usually, they are diffusely scattered and greatly found in organs such as lungs (alveolar macrophages) and may act as a filter for particular agent. These cells constitute the critical mainstay during the antigenic infection leading to eliminations of the infected cells. Serious tissue destructions and pulmonary edema suggest that emphysema and interstitial pneumonia may lead to a respiratory failure and could provide direct evidence for death (Uilenberg 1981; Irvin and Morrison, 1987; Tageldin *et al*., 2005; El Imam, 2015; El Imam and Taha, 2015). Consequently, we may speculate that *T. lestoquardi* is a respiratory disease.

The results of the PCR confirmation certainly proved that all animals that showed macroscopic and microscopic alterations were infected with *T. lestoquardi* and no postmortem and histopathological changes were reported in the non-infected control animals. The present study may represent a precise study on macroscopic and microscopic findings of pathogenic *Theileria* infecting small ruminant.

5. Conclusion

The prominent and remarkable macroscopic and microscopic lesions during the different courses of the disease were seen in the lungs. The serious pulmonary tissues destructions suggest that emphysema and interstitial pneumonia may lead to a respiratory failure and could provide evidence for death. The present investigation gives clear evidence that native Sudanese sheep are highly susceptible to *T. lestoquardi* infections. Therefore, the disease will be lethal to sheep population if they are exposed to infected ticks in endemic area, such as Atbara, warranting more attention to ticks control strategy and vaccine production.

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Design, Cloning and *In silico* Analysis of Efficient siRNA-inducing Cassette for Silencing Wheat γ-gliadins

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Abstract

Gluten is an important protein source for human beings and a wide diversity of foods has been developed to take advantage of this protein in wheat flour. However, some individuals, suffering from Celiac Disease (CD), cannot tolerate proline and glutamine-rich gluten peptides including γ-gliadins. A life-long gluten free diet is the only known effective treatment for such patients. However, the sensitivity intension in CD patients can be controlled by RNA interference (RNAi) technology. The main aim of the present study is to develop an efficient and specific siRNA-inducing cassette, as a first and critical step for an effective targeting of mRNAs of wheat γ-gliadins. To achieve this aim, we have followed the strategy based on 200 bp in sense and antisense orientation with the ~160bp sequence of none potent siRNA-containing region from γ-gliadin gene as a spacer in between. The endosperm-specific γ-gliadin promoter was sub-cloned into upstream the cassette. The nucleotide alignment results validated the sequence data of γ-gliadin promoter and the direct inserts with high homology identities of 99% and 99.97%, respectively. Here, six potential and consecutively arranged-siRNA sites were predicted using computational approaches. All of these sites covered the inverted repeats region with high efficacy and performance values for triggering RNAi.

Keywords: Celiac disease, Common wheat, γ-gliadins, *In silico* analysis, siRNA-inducing cassette.

1. Introduction

Gluten is a complex mixture of protein components comprising the gliadins and glutenins in wheat and equivalent proteins in barley and rye (Rosell *et al.*, 2014). Gliadins can be subdivided into four sub-fractions: α/β, γ and ω-gliadins, whereas glutenins consist of low and high molecular weight glutenins (LMW, HMW) (Colomba and Gregorini, 2012). The γ-type gliadins that contribute to the visco-elastic properties of the dough are mainly heterogeneous collection of 30-78 kDa monomeric proteins with poor solubility in dilute salt solutions and good solubility in 70% ethanol (Guo *et al.*, 2012; Shewry and Tatham, 1990; Wieser, 2007). These peptides account for approximately 12% of the flour proteins of the hexaploid wheat (Dupont *et al.*, 2011). They are encoded by clusters of linked genes, present at the Gli-1 loci on the short arms of the respective homologous group 1 chromosomes of hexaploid bread wheat (*Triticum aestivum* L.) and are tightly linked to the Glu-3 (LMW glutenins) and Gli-3 (ω-gliadins) loci (Dupont *et al.*, 2011; Gao *et al.*, 2007). The number of different γ-gliadin genes in the genome of bread wheat was estimated at 15-40 copies and these can be clustered into four up to eleven groups (Payne, 1987; Qi *et al.*, 2009). γ-gliadins show a strong association with Celiac Disease (CD), a widely prevalent (0.5-1% of the general populations) chronic inflammatory condition of small intestine triggered by the ingestion of gluten fractions derived from wheat, barley and rye (Colomba and Gregorini, 2012; Ferretti *et al.*, 2012; Van den Broeck *et al.*, 2009). Since the several sets of CD-epitope cores (9-mer peptides) are located in the first variable domain R1 (domain II) region of γ-gliadins, they could be great initiators of CD (Anderson *et al.*, 2012; Salentijn *et al.*, 2012). These epitopes, generally with their highly proline (P) and glutamine (Q) content, are perfect substrate for transglutaminase reaction of tissue transglutaminase 2 (tTG2) enzyme, which are critical for the creation of active T-cell epitopes

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Abbreviations :CD Celiac Disease, CTAB Cetyl Triethyl Ammonium Bromide, dNTPs deoxy nucleoside5´-triphosphates, dsRNA double strand RNA, hpRNA hairpin RNA, HMW High Molecular Weight, IPTG Isopropyl β-D-1-thiogalactopyranoside, kD kilo Dalton, LB Luria Bertani, LMW Low molecular weight, MFE Minimum Free Energy, mRNA messenger RNA, NCBI National Center for Biotechnological Information, PCR Polymerase Chain Reaction ,pssRNAit plant short small RNA interfering tool, PTGS Post Transcriptional Gene Silencing, RISC RNA-Induced Silencing Complex, RNAi RNA interference, siRNA small interfering RNA, UPE Unpaired Energy, `X-gal 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside, (Anderson *et al.*, 2012; Kim *et al.*,2004; Meresse *et al.*, 2012; Salentijn *et al.*, 2012). Although a lifelong gluten-exclusion diet is the only available and effective treatment for CD patients, complying with a gluten-free diet is tedious and affects the patients' quality of life (Gil-Humanes *et al.*, 2014). According to some reports, most of down-regulation of gluten proteins in wheat and other cereals has been based on RNAi technology (Rosell *et al.*, 2014). RNA silencing of specific individual gliadins or groups of prolamins may therefore, be of interest in relation to determining their role in both grain-processing properties and in triggering celiac disease (Gil-Humanes *et al.*, 2008).

Since RNAi is a sequence-specific RNA degradation system that is conserved in a wide range of organisms, it was rapidly favored as a powerful research tool for the Post-Transcriptional Gene Silencing (PTGS) in plants. The mechanism of RNAi is similar in all eukaryotes and is triggered by double strand RNA (dsRNA) molecules through a two-step mechanism (Gil-Humanes *et al.*, 2010). RNAi gene silencing protects the organism's genome from transposons and viruses as well as a part of the defense system in plants (Kemp *et al.*, 2013; Nicolas *et al.*, 2013). In recent decade, with realizing the importance of RNAi technology for effective down-regulating in the expression of a plant gene of interest, the various specific RNAi-based vectors for the construction and the expression of hairpin-like RNA constructs in plant cells have been developed. The expression of an RNAi-inducing cassette results in a dsRNA molecules composed of two distinct regions: a single-stranded loop, encoded by the spacer region and a double strand stem encoded by an inverted repeats (Edman and Waterhouse, 2011).

The main aim of the present work was to generate the tissue specific and effective siRNA-inducing cassette, as a first and critical step for effective targeting of γ-gliadin transcripts from *T. aestivum*. Therefore, an effort has been exerted to identify potential siRNA candidates for silencing the target messenger RNA (mRNA) using a computational approach. Furthermore, we attempted to predict the secondary structure formed between siRNA and the target mRNAs.

2. Materials and Methods

2.1. Sequence retrieval and bioinformatics analysis

The total number of 7 γ-gliadin promoter and 140 mRNA sequences was identified as orthologous of wheat γ-gliadins genes from GenBank at the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/BLAST/). CLUSTALW program (accurate) was used to carry out nucleotide sequence alignments separately among the sequences of γ-gliadin promoter and the total mRNA sequences of wheat γ-gliadin genes (McWilliam *et al.*, 2013). The accession numbers of the γ-gliadin promoter, used for sequence alignment, were as follows: Wheat transformation vector pGhp-omega/alpha/beta, HM352558; *T. aestivum*, FJ231103, FJ234648, FJ234649, EF426565, and AF234647; Wheat γ-gliadin, M36999. The accession numbers of γ-gliadins mRNA from *T. aestivum*, used for sequence alignment analysis, were as follows: A total 103 complete gene sequences, including HQ631424, JN849087-96, KF412602-614, KC715955-67, KC715971-91, KC715996, KC716000-6004, JQ943400-406, JN849087, JN849090-93, AY338386-390 and FJ006589-623; a total of 37 pseudogene sequences, including FJ006678-83, KF412615-21, KC715968-70, KC715992-95, KC715997-999, KC716005-011, JN849083-86, JN849088, JN849089, and JN849094.HM352558 and FJ006593 accession numbers were selected for designing the primer sets based on alignment results respectively belonged to γ-gliadin promoter and γ-gliadin mRNA sequences.

2.2. Plant, plasmid, enzymes, and chemicals

Wheat (*T. aestivum* L.) plants were grown in a pod in a greenhouse condition. The pTG19-T cloning vector (Vivantis, USA) derived from pTZ19-R vector (Accession no. Y14835.1) was used as a plasmid backbone for all cloning purposes. *Escherichia coli* strain DH5α (Invitrogen, USA) was used as a host strain for molecular cloning. Restriction enzymes, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) and Isopropyl β-D-1-thiogalactopyranoside (IPTG) were obtained from Thermo Fisher Scientific Inc. (USA). All the other chemicals were molecular biology grade (Merck, Germany). The sequence of the γ-gliadin siRNA-inducing cassette is available at Gene Bank under accession number KT732419.

2.3. Genomic DNA extraction

After a 24-hour darkness treatment for a full break-down of the cellular starch content, the total genomic DNA was isolated from 2-3 leaves growth stage using Cetyl Trimethyl Ammonium Bromide (CTAB) reagent method (Doyle and Doyle, 1987). The concentration of genomic DNA was measured spectrophotometrically and its quality was checked by 0.8% agarose gel electrophoresis.

2.4. Primer design and PCR amplification

The specific primer sets for γ-gliadin promoter were synthesized from accession number HM352558, direct and inverted fragments from FJ006593. All primers were designed using Primer3 software at NCBI and analyzed by Oligo analyzer software (version 7.56).The restriction sites, arbitrarily defined (underlined letters), were added at the 5'-ends of forward and reverse primer sets (Table 1).

Table 1. Primers used for the synthesis of the siRNA-inducing cassette

Primer + The enzyme added at the 5′-end	Description	Sequence 5′to 3′
γ-GliPromoter_F+ *Hind*III	Forward primer for the synthesis of γ-gliadin promoter	ATAAGCTTTTCCAGAAAAAACTTTGCTA
γ-GliPromoter_R+ *Pst*I	Reverse primer for the synthesis of γ-gliadin promoter	ATCTGCAGGGTGGATTTGCGTTAACTAC
γ-GliDirect_F+ *Kpn*I	Forward primer for the synthesis of the 360bp γ-gliadin direct fragment	ATGGTACCGCCACAACAACAACCAGTCC
γ-GliDirect_R+ *Nhe*I	Reverse primer for the synthesis of the 360bp γ-gliadin direct fragment	ATGCTAGCTCTTGCAGGGGTTCATCTGT
γ-GliInverted_F+ *Xba*I	Forward primer for the synthesis of the 200bp γ-gliadin inverted fragment	ATTCTAGAAACAAACATTCCCCCAACGA
γ-GliInverted_R+ *Pst*I	Reverse primer for the synthesis of the 200bp γ-gliadin inverted fragment	ATCTGCAGTGACTGAATCGCCGGTTGT

2.5. Molecular cloning

First PCR-amplified promoter and direct fragment were purified by gel purification kit (Bioneer, South Korea; cat. no. k-3035) according to the manufacturer's instructions. Then, they were TA-cloned into linear pTG19-T cloning vector separately by T4 DNA ligase (200u/µl, Vivantis, USA). For screening, after the recovery of bacteria on antibiotic-free Luria-Bertani (LB)-liquid culture, the cells were plated on LB-agar medium (peptone 1% (w/v), yeast extract 0.5% (w/v), NaCl 1% (w/v), agar 1.2% (w/v)) containing ampicillin (100µg/ml), X-gal (100µg/ml) and IPTG (1mM). The positive clones for direct and promoter inserts were sequenced. At the second step, the inverted fragment was amplified from plasmid containing direct fragment. At the final step, the inverted and promoter fragments were sub-cloned at upstream of the direct insert and the cassette, respectively. The cassette was validated by *Hind*III/*Nhe*I double restriction digestion and verified by sequencing. T7 promoter primer (TAATACGACTCACATTAGGG), presented in Figure 2, was used for sequencing.

2.6. Determination of multiple siRNA candidates

So far, a number of experimental rules on siRNA duplex features have been frequently reported. These include the asymmetry rules for siRNA duplex ends, high A/U content at the 5'-end of the antisense strand, high G/C content at the 5'-end of sense strand, 30-50% GC content, thermodynamic properties in term of the secondary structures, accessibilities of siRNA and target mRNA of gene(s) of interest (Nur *et al.*, 2014). Therefore, the plant short small RNA interfering tool (pssRNAit web tool), which provides a functional and an off-target minimized siRNA design, was used to *in-silico* identifying efficient siRNAs candidates in the target mRNAs (Http://plantgrn.noble.org/pssRNAit/). The sequence data of the direct insert were used to determine potential siRNA sites in the target repeat.

2.7. GC calculation and off-target alignment

The DNA/RNA GC content calculator (http://www.endmemo.com/Tools/Biology) was used to calculate the GC content of the predicted siRNA. For checking any off-target sequence resemblance in other non-targeted organism's genome, the BLAST tool (http://www.ncbi.nlm.nih.gov/blast) was used against the whole GenBank database by applying expected thresholds value 10 and BLOSUM 62 matrix as parameter (Nur *et al.*, 2014).

2.8. Prediction of the secondary structure

The UNAfold program module in mfold web server (http://mfold.rna.albany.edu) was used to predict the secondary structure formed between mRNA and RNAi guide strands (Zuker, 2003).

3. Results and Discussion

Since RNAi technology is being used for down-regulating wheat γ-gliadin genes, an efficient inducing cassette is needed (Gil-Humanes *et al.*, 2010). To reach this aim, it is important to develop the efficient hpRNA-inducing cassette as a critical triggering to target mRNAs of wheat γ-gliadins. Therefore, we have designed and cloned the γ-gliadin-specific siRNA-inducing cassette to produce a ~200bp stem with six siRNAs sites harboring a ~160bp loop without potent siRNAs.

3.1. Identification of potent siRNAs

According to previous reports, the presence of some specific and effective siRNA candidates in dsRNA region could be assuming as a critical point for triggering RNAi. However, only a small proportion of randomly selected siRNAs are potent, and a large variation in the efficiency of siRNAs for different sites on the same target commonly was observed (Yan *et al.*, 2009). In the present work, the total number and nucleotide sequence of the efficient siRNA sites, located in the sequence data of ~360 bp direct fragment, was determined using pssRNAit server tool (Table 2). Twenty out of the total of thirty-two siRNA candidates were selected with 7-10 and 8-10 scores for off-target accessibility and efficiency values, respectively. For further validation of reduced off-targets, all siRNA are subjected to BLASTn. As it is important to ensure whether RNAi binds RISC or not, results presented, as in Table 2, prove the successful incorporation of siRNA antisense strand with RISC. All the siRNA holds GC content within 36-48% were determined by GC content calculator. Lower GC content leads to stronger inhibitory effects and 30-57% of GC content is considered sufficient for the execution of RNAi's action (Nur *et al.*, 2014; Bret *et al.*, 2005; Liu *et al.*, 2013). Consequently, after the analysis of all siRNA candidates, six potential siRNAs, which were consecutively arranged in the target repeats, were considered as the efficient candidates for effective triggering γ-gliadin target mRNAs.

3.2. The secondary structures of siRNA-target mRNA duplexes

Secondary structures of RNAi do not only provide a convenient and computationally tractable estimation to structures but they also provide them to the thermodynamics of RNA-RNA interaction (Nur *et al.*, 2014). Furthermore, the secondary structure of RNAi can be involved in the RISC cleavage. Therefore, the prediction of the siRNA-mRNA secondary structure is the most important factor to select the efficient guide strand of siRNA sites for the effective triggering of RNAi (Bret et al., 2005; Liu et al., 2013). UNAfold software predicts the most stable secondary structure of an oligonucleotide by minimizing folding free energy. The secondary structures of the selected efficient siRNAs are shown in Figure 1(A)-(F). The ΔG-binding of siRNA-mRNA duplexes with minimum free energy are shown in Table 2.

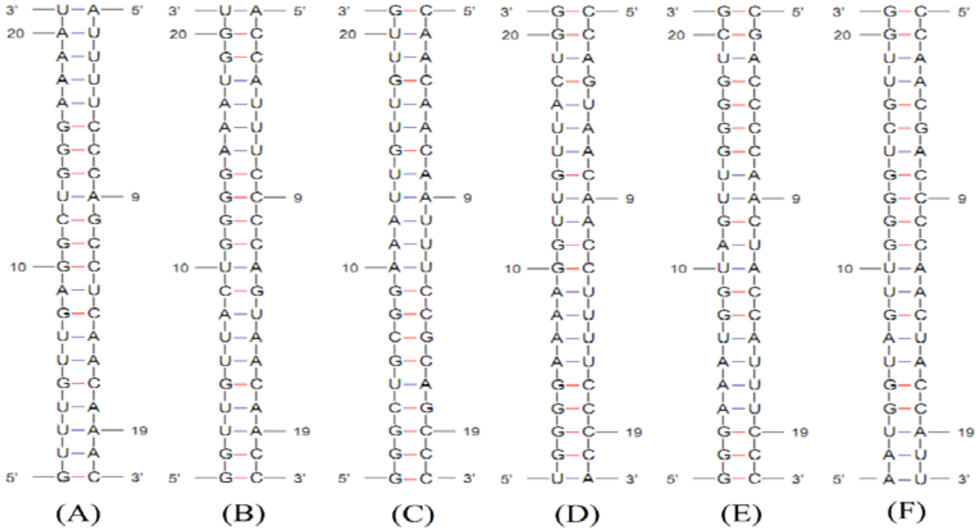

Figure1. (A)-(F). Schematic diagram of the secondary structures of duplexes between antisense strands of the selected six siRNAs (left strands) and the target mRNA of wheat γ-gliadins. The folded structures are predicted by minimizing folding free energy at the speciifed temperature (28 °C)

Table 2. Effective siRNA molecules with target accessibilities, efficiency, GC percentage, free energy of binding with target

Target No.	Location of target within mRNA	Sequence of siRNA duplex	GC%	ΔG binding (k/Cal)	UPE [a]	siRNA Efficiency [b]	RISC binding antisense scores [c]	RISC binding sense scores [c]
01	256-276	GUUUGUUGAGGCUGGGAAAAU | | | | | | | | | | | | | | | | | | | UACAAACAACUCCGACCCUUU	42.85	-38.47	7.08	9.2	0.4	0.4
02	284-304	GGUUGUUACUGGGGAAAUGGU | | | | | | | | | | | | | | | | | | | UUCCAACAAUGACCCCUUUAC	47.61	-38.49	8.43	8.18	0.4	0.33
03	165-185	GGGCUGCGGAAAUUGUUGUUG | | | | | | | | | | | | | | | | | | | ACCCCGACGCCUUUAACAACA	50.08	-38.86	8.37	7.97	0.24	0.24
04	304-325	UGGGGAAAAGGUUGUUACUGG | | | | | | | | | | | | | | | | | | | CGACCCCUUUUCCAACAAUGA	47.60	-38.02	8.58	7.59	0.33	0.33
05	219-239	GGGAAAUGGUAGUUGGGGUCG | | | | | | | | | | | | | | | | | | | ACCCCUUUACCAUCAACCCCA	47.98	-30.76	9.34	7.41	0.24	0.14
06	268-289	AAUGGUAGUUGGGGUCGUUGG | | | | | | | | | | | | | | | | | | | CUUUACCAUCAACCCCAGCAA	47.61	-30.42	10.2	7.01	0.33	0.24

a Off-target accessibility or unpaired energy (UPE):the energy required to open mRNA secondary structure around target site is represented by UPE score in the range of 0-25 accessible scores. The less UPE score means the more possibility that siRNA is able to contact with target mRNA, which leads to the less off-target accessibility (Dai and Zhao, 2011).

b siRNA efficiency: Efficiency denotes the effectiveness of designed siRNA to silence transcripts. The efficiency range can vary from 0-10, higher the value greater silencing of transcript (Dai and Zhao, 2011).

c RISC binding score including sense and antisense strands indicate the binding abilities of each siRNA strands to RISC complex for triggering RNAi. The binding score range vary from - 0.2 to 2 values and the higher score shows an efficient binding to RISC complex.

3.3. Cloning and screening of the specific siRNA-inducing cassette

To achieve such a high performance of siRNA-inducing molecules, the endosperm-specific γ-gliadin promoter was used for a specific and a strong expression in endosperm, representing approximately 80% total grain proteins (Rosell *et al.*, 2014; Dupont *et al.*, 2011; Gil-Humanes *et al.*, 2010; Piston *et al.*, 2013; Piston *et al.*, 2009). The alignment result of the γ-gliadin promoter insert showed a maximum homology with the γ-prolamin promoter sequences from public databases and the previously reported γ-gliadin promoter with efficient performance of 98% and 100%, respectively. The nucleotide alignment of 360bp direct insert with 140 γ-gliadin mRNA sequence data were performed, and they showed average homology identities of 99.97% (accession numbers of data presented at materials and methods). According to the high affinity in binding RISC to antisense strand of double stranded siRNAs, the orientation of direct and inverted fragments was adjusted as in Figure 2. Consequently, the promoter fragment was sub-cloned at upstream of the inverted fragment, which was considered as a sense strand.

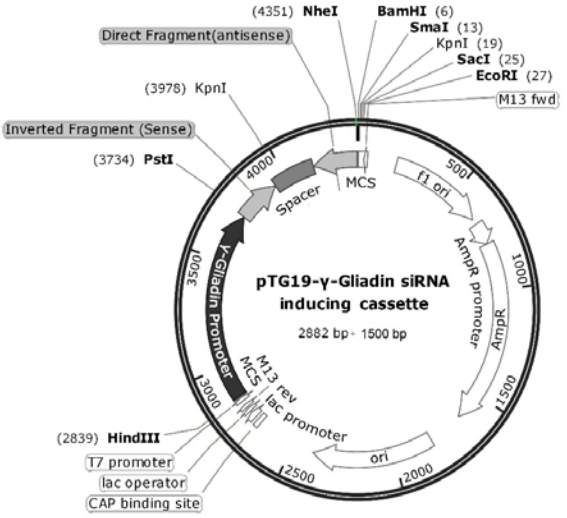

Figure 2. Schematic presentation of wheat γ-gliadins-specific hpRNA-inducing cassette cloned into pTG19-T cloning vector with ~4350bp-long.

In the cloning of the direct fragment, the correct colonies were screened out by standard blue/white screening system. The DNA plasmid with 360bp direct fragment was confirmed by *Nhe*I restriction digestion (Figure 3(A)). The inverted fragment was sub-cloned during a ligation-restriction step to the plasmid DNA with direct insert by double digestion of *Pst*I/*Xba*I restriction sites. In Figure 3(B), the plasmid DNA, containing the inverted repeats (~4350bp in length, Lane2), was distinguished from the plasmid harboring only direct fragment (~3250bp in length, Lane3). The final size of DNA plasmids harboring the siRNA-inducing cassette (~1500bp in length) was validated by *Hind*III/*Nhe*I double restriction digestions (Figure 3(C)).

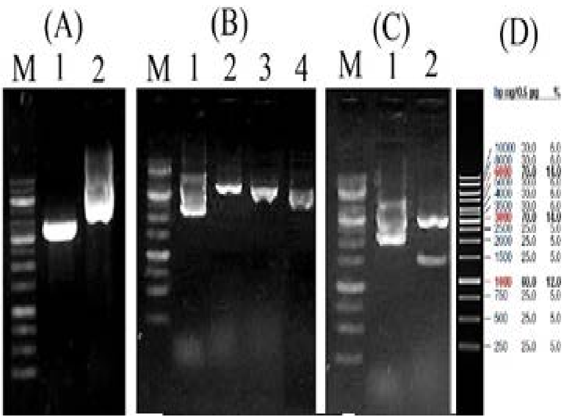

Figure3. Step by step analysis of developing siRNA-inducing cassette.(A)Screening of plasmid DNA with 360bp direct fragment (Lane 2) digested by *Nhe*I restriction enzyme (Lane 1) and validated based on size by gene ruler 1kb DNA ladder (Lane M).(B)The exact size of the plasmid DNAs with direct insert (Lane 4) and the inverted repeat (Lane 1) were validated by gene ruler 1kb DNA ladder (Lane M). The 4350bp plasmid DNA with two inserts (Lane 2) was distinguished based on size from the 3250bp plasmid DNA containing direct fragment (Lane 3). (C) The full cassette construct was excised from the recombinant DNA plasmid (Lane 1) by *Hind*III/*Nhe*I double restriction digestion (Lane 2). (D) Illustration of gene ruler 1kb DNA ladder (Thermo Scientific Co., USA) with three sharp reference bands (6000, 3000 and 1000 bp) loaded on 0.8% agarose gel by Red Safe™ 5% (v/v).

4. Discussion

RNAi technologies make it possible to effectively down-regulate the target mRNA of γ-gliadin multigenes from wheat, which causes celiac disease symptoms in genetically predisposed individuals. Thus, we have designed and developed an efficient and a specific siRNA-inducing cassette as a critical step for targeting wheat γ-gliadins. We have followed the strategy based on 200bp in sense and antisense orientation with a ~160bp sequence of none potent siRNA-containing region of γ-Gliadin direct insert as a spacer in between. To achieve the high performance siRNA-inducing cassette, the previously reported endosperm-specific γ-gliadin strong promoter was used to drive siRNA-inducing cassette. The nucleotide alignment results validated the sequence data of γ-gliadin promoter and direct inserts with high homology identities of 99% and 99.97%, respectively. Here, six potential and consecutively arranged-siRNA sites were predicted in the inverted repeat region using computational approaches. The consequences of the present study demonstrated that these potential siRNA sites have high efficacy and performance values for triggering of RNAi.

Acknowledgements

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Resistance of *Callosobruchus maculatus* (Fabricius) (Coleoptera: Bruchidae) Populations in Nigeria to Dichlorvos

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Abstract

The resistance status of Nigeria populations of *Callosobruchus maculatus* (Fab.) to dichlorvos (2,3 – Dichlorovinyl dimethyl phosphate {DDVP}) was investigated in the present study. Bruchids were obtained from five different locations spread across three South-Western states of Nigeria. These include Akure (Ondo state), Ikare-Akoko (Ondo state), Ikere-Ekiti (Ekiti state), Ijan-Ekiti (Ekiti state) and Ibadan (Oyo state). A susceptible laboratory culture of *C. maculatus* served as the reference population. Bruchid populations were exposed to filter papers impregnated with DDVP (concentration range: 0.00001% - 0.01%) and the mortality was assessed after 3, 6, 24 and 48 hours post-treatment. Bruchid mortality varied across locations, DDVP concentration and exposure time. Bruchids obtained from Ibadan required the highest concentration (LC_{95}: 185.418 mg ml^{-1}) of DDVP, hence they have the highest resistance factor (RF) (RF_{95}: 1483.30); while their counterparts from Ijan-Ekiti required the lowest concentration of DDVP (LC_{95}: 0.242 mg ml^{-1}) with the lowest RF (RF_{95}: 1.94). The present study reveals diverse levels of resistance to DDVP in Nigerian populations of *C. maculatus*.Hence, there is a need for resistance management strategies on the use of DDVP and other organophosphate insecticide in its class across various Nigerian states to minimize cost and health risk implications that could arise from insecticide resistance.

Keywords: **:** Resistance, DDVP, Populations, *Callosobruchus maculatus*, Mortality.

1. Introduction

In spite of the widespread public concern about most synthetic insecticides on human health and environment, they are still heavily used and considered as the most effective method of controlling stored product pests in most nations, particularly for a large scale storage (Isman, 2000; Gbaye and Holloway, 2011). In Nigeria, for instance, the chemical control method is the most commonly used for pest management (Chedi and Aliyu, 2010). Even though the research on the use of botanical pesticides has gained prominence over the years, myriad of problems, such as relatively slow action, variable efficacy, instability in the environment, disagreeable odour, poor water solubility and inconsistent availability among others have trivialized the use of botanical pesticides against the newest generations of synthetic insecticides (Moretti et al., 2002; Isman and Grieneisen, 2014).

Synthetic insecticides, such as malathion, aluminium phosphide, pirimiphos-methyl, dichlorvos (dichlorovinyl dimethyl phosphate- DDVP), deltamethrin, cypermethrin and carbaryl, among others, are being used for controlling stored product pest either as fumigants or contact insecticides (Desmarchelier, 1994; Zettler et al., 1997; Gbaye et al., 2012; Perveen and Khan, 2014). Although some of these chemicals have been banned in developed countries, some of them, especially dichlorvos (DDVP), are still being used to control households and stored products insects in some developing countries, including Nigeria (Chedi and Aliyu, 2010). DDVP is an organophosphate which has exhibited high efficacy against storage insect pests, both as contact and stomach poison (Rahman, 1990; Lotti, 2001; Booth et al., 2007).

Nigeria, being the largest producer and consumer of cowpea in the world as it accounts for 61% production in Africa and 58% worldwide, usually record huge post-harvest losses due to the debilitating effect of Callosobruchus maculatus on cowpea seeds (Singh and Ntare, 1985; IITA, 2010). Most cowpea merchants in Nigeria rely on the use of chemicals, such as DDVP to control this insect pest. Due to the high level of illiteracy among local farmers and post-harvest handlers of cowpea in Nigeria, pesticides are indiscriminately applied for insect pest control. Oyeniyi et al. (2015a) opined that mismanagement of any form of insecticide can lead to resistance and loss of efficacy overtime. Insecticidal resistance, due to the failure of most chemicals to control insect pest, has also been implicated in the loss of food

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worth several billions of dollars globally (Elzen and Hardeen, 2003). Singh and Ntare (1985) reported that a 5% annual production loss to *C. maculatus* in Nigeria alone would cost about $100 million. Therefore, the knowledge of insecticidal resistance status is needed to reduce the huge post-harvest losses associated with cowpea production and utilization in Nigeria.

The effect of vegetation on the resistance of cowpea Bruchids in Nigeria to pirimiphos-methyl has been investigated (Odeyemi et al., 2006). However, investigating the possible resistance of *C. maculatus* to DDVP in Nigeria has not been reported. Hence, the present study assessed the resistance status of *C. maculatus* populations in Nigeria to DDVP (2,3 – Dichlorovinyl dimethyl phosphate).

2. Materials and Methods

2.1. Insect Collection and Culture

The present study was carried out in the Biology Laboratory, Federal University of Technology Akure, Ondo state, Nigeria. *Callosobruchus maculatus* was obtained from infested cowpea seeds sourced from stores in five different locations across three states in Nigeria. These include: Akure (Ondo state), Ikare-Akoko (Ondo state), Ikere-Ekiti (Ekiti state), Ijan-Ekiti (Ekiti state) and Ibadan (Oyo state). Clean cowpea seeds (Sokoto white cultivar) were disinfested in the freezer at -18°C for two weeks, and prior to use, they were allowed to equilibrate in the laboratory for three days at ambient temperature and humidity (28±2ºC and 88±5%) to prevent mouldiness. Bruchid samples from each location were introduced into 2.5 litres transparent plastic containers containing 200g of disinfested cowpea seeds and reared to F_1 generation. A susceptible laboratory culture of *C. maculatus* was obtained from Research Laboratory, Biology Department, Federal University of Technology, Akure, Ondo State, Nigeria. The laboratory culture served as the reference population and it was not exposed to synthetic insecticides or botanicals.

2.2. Preparation of the Chemical

2, 3-Dichlorovinyl dimethyl phosphate, DDVP, (Sniper® 1000EC) used for the experiment was obtained from an Agrochemical store in Ibadan, Oyo state, Nigeria. Various concentrations of the insecticide (DDVP) were prepared by dilution with acetone. The concentrations used were 0.00001%, 0.0001%, 0.0005%, 0.001%, 0.005% and 0.01%, while acetone only (0.0%) served as the control treatment.

2.3. Experimental Procedures

Impregnated filter paper technique, described in FAO method 15 (Anonymous, 1974) and modified for bruchids by Tyler and Evans (1981), was used to evaluate the resistance of *C. maculatus* to DDVP. Whatman's No.1 filter papers (110mm diameter) were treated with the varying concentrations of DDVP listed above. 0.5ml of a concentration was applied to a filter paper with the aid of 1-ml syringe and the paper was allowed to air-dry for acetone to evaporate. Twenty unsexed adult *C. maculatus* were released onto each treated filter paper and covered with Petri-dish. Each treatment was replicated three times. Bruchids mated and laid eggs soon after adult emergence, hence the mortality was observed after 3, 6, 24 and 48 hours post-treatment. Bruchids were confirmed dead if there was no response when their abdomen is gently prodded with a needle. This procedure was done separately for all the Bruchid populations sampled and the various concentrations of the insecticides.

2.4. Statistical Analysis

Abbott formula (1925) was used to correct all data on adult mortality counts using control mortality. The data were subjected to analysis of variance (ANOVA) at p<0.05 and the treatment means were separated using Tukey's Test. Data on adult mortality were subjected to probit analysis to determine the concentration of DDVP required to achieve 75% (LC_{75}) and 95% (LC_{95}) mortality in each sampled population (Finney, 1971). Data analysis was performed with Statistical Package for Social Sciences (SPSS) 17.0 software.

$$\text{Resistance Factor (RF)} = \frac{\text{Lethal concentration of each population}}{\text{Lethal concentration of laboratory population}}$$

The resistance factor of each population was calculated from the lethal concentrations using the expression below:
(Modified from Oyeniyi et al., 2015a)

3. Results and Discussion

3.1. Response of C. maculatus Populations to DDVP

Tables 1-6 show the response of *C. maculatus* populations obtained from different locations to different concentrations of DDVP. Bruchid response varied with different concentrations and exposure times. There was a significant effect of DDVP concentration on the mortality of *C. maculatus* after a 3-hour exposure in all the populations (Akure: $F_{6,14}$ = 11.600, P<0.0001; Ikare-Akoko: $F_{6,14}$ = 17.694, P<0.0001; Ikere-Ekiti: $F_{6,14}$ = 7.833, P = 0.001; Ijan-Ekiti: $F_{6,14}$ = 5.333, P = 0.005; Ibadan: $F_{6,14}$ = 3.804, P = 0.018), except for laboratory population ($F_{6,14}$ = 1.373; P = 0.291). Similarly, at 6, 24 and 48 h post-treatment, significant effects (P<0.0001) of DDVP concentration were observed in the bruchids. However, the highest effect was observed at 48 h post-treatment when compared to those exposed at other durations. After 48 hours of exposure, bruchids, obtained from all the locations, showed complete mortality (100%) at 0.005% and 0.01% concentrations, except for bruchids from Ibadan which had 100% mortality only at the highest experimental concentration (0.01%). For the laboratory population, complete mortality was observed at 0.001%, 0.005% and 0.01%.

Table 1. Effect of DDVP on the mortality (% mean ± S.E.) of *C. maculatus* population from Akure

Concentration %	Duration (Hours)			
	3	6	24	48
0.0	$0.00 \pm 0.00^{a(a)}$	$0.00 \pm 0.00^{a(a)}$	$0.00 \pm 0.00^{a(a)}$	$0.00 \pm 0.00^{a(a)}$
0.00001	$0.00\pm0.00^{a(a)}$	$0.00\pm0.00^{a(a)}$	$21.65\pm1.65^{b(b)}$	$36.65\pm4.40^{b(c)}$
0.0001	$0.00\pm0.00^{a(a)}$	$0.00\pm0.00^{a(a)}$	$30.00\pm5.75^{b(b)}$	$78.13\pm5.75^{b(c)}$
0.0005	$5.00\pm2.90^{b(a)}$	$18.35\pm6.00^{b(b)}$	$45.00\pm5.75^{bc(c)}$	$87.55\pm5.75^{c(d)}$
0.001	$7.00\pm2.98^{ab(a)}$	$20.00\pm2.90^{b(b)}$	$65.00\pm5.75^{cd(c)}$	$91.36\pm2.90^{d(d)}$
0.005	$10.00\pm2.98^{b(a)}$	$30.00\pm2.90^{c(b)}$	$73.35\pm12.00^{c(c)}$	$100.00\pm0.00^{d(d)}$
0.01	$18.35\pm1.65^{c(a)}$	$35.00\pm2.90^{c(b)}$	$76.65\pm6.00^{c(c)}$	$100.00\pm0.00^{d(d)}$

Mean values followed by the same letter(s) are not significantly different (P>0.05) by Tukey's Test. Letters immediately following the means are for vertical comparison while letters in parenthesis are for horizontal comparison.

Table 2. Effect of DDVP on the mortality (%mean ± S.E.) of *C. maculatus* population from Ikare Akoko

Concentration %	Duration (Hours)			
	3	6	24	48
0.0	$0.00\pm0.00^{a(a)}$	$0.00\pm0.00^{a(a)}$	$0.00\pm0.00^{a(a)}$	$0.00\pm0.00^{a(a)}$
0.00001	$0.00\pm0.00^{a(a)}$	$0.00\pm0.00^{a(a)}$	$25.00\pm2.90^{b(b)}$	$45.00\pm1.91^{b(c)}$
0.0001	$1.65\pm1.65^{a(a)}$	$3.35\pm1.65^{a(a)}$	$38.35\pm3.25^{c(b)}$	$51.65\pm4.05^{b(c)}$
0.0005	$3.35\pm1.65^{a(a)}$	$10.00\pm5.00^{a(a)}$	$40.00\pm5.00^{c(b)}$	$56.65\pm8.23^{b(c)}$
0.001	$13.35\pm3.35^{b(a)}$	$26.65\pm4.40^{b(b)}$	$51.65\pm4.40^{d(c)}$	$96.65 \pm0.00^{c(d)}$
0.005	$20.00\pm2.90^{bc(a)}$	$50.00\pm5.75^{c(b)}$	$70.00\pm2.90^{e(c)}$	$100.00\pm1.91^{c(d)}$
0.01	$26.00\pm2.90^{c(a)}$	$55.00\pm5.75^{c(b)}$	$71.65\pm7.25^{e(c)}$	$100.00\pm0.00^{c(d)}$

Mean values followed by the same letter(s) are not significantly different (P>0.05) by Tukey's Test. Letters immediately following the means are for vertical comparison while letters in parenthesis are for horizontal comparison.

Table 3. Effect of DDVP on the mortality (%mean ± S.E.) of *C. maculatus* population from Ikere Ekiti

Concentration %	Duration (Hours)			
	3	6	24	48
0.0	$0.00 \pm 0.00^{a(a)}$	$0.00 \pm 0.00^{a(a)}$	$0.00 \pm 0.00^{a(a)}$	$0.00 \pm 0.00^{a(a)}$
0.00001	$0.00\pm0.00^{a(a)}$	$0.00\pm0.00^{a(a)}$	$13.35\pm1.65^{b(b)}$	$45.00\pm7.25^{b(c)}$
0.0001	$0.00\pm0.00^{a(a)}$	$0.00\pm0.00^{a(a)}$	$20.00\pm5.75^{b(b)}$	$46.67\pm7.25^{b(c)}$
0.0005	$1.65\pm1.65^{a(a)}$	$13.35\pm3.35^{b(b)}$	$43.35\pm6.00^{c(c)}$	$91.65\pm1.65^{c(d)}$
0.001	$5.00\pm2.90^{a(a)}$	$20.00\pm2.90^{bc(b)}$	$50.00\pm5.75^{c(c)}$	$91.65\pm1.65^{c(d)}$
0.005	$10.00\pm2.90^{ab(a)}$	$23.00\pm7.65^{bc(b)}$	$71.65\pm4.40^{d(c)}$	$100.00 \pm0.00^{c(d)}$
0.01	$11.65\pm1.65^{ab(a)}$	$30.00\pm5.75^{c(b)}$	$73.65\pm4.40^{d(c)}$	$100.00\pm0.00^{c(d)}$

Mean values followed by the same letter(s) are not significantly different (P>0.05) by Tukey's Test. Letters immediately following the means are for vertical comparison while letters in parenthesis are for horizontal comparison.

Table 4. Effect of DDVP on the mortality (%mean ± S.E.) of *C. maculatus* population from Ijan Ekiti

Concentration %	Duration (Hours)			
	3	6	24	48
0.0	$0.00\pm0.00^{a(a)}$	$0.00\pm0.00^{a(a)}$	$0.00\pm0.00^{a(a)}$	$0.00\pm0.00^{a(a)}$
0.00001	$0.00\pm0.00^{a(a)}$	$0.00\pm0.00^{a(a)}$	$21.65\pm2.00^{b(b)}$	$48.35\pm4.40^{b(c)}$
0.0001	$0.00\pm0.00^{a(a)}$	$0.00\pm0.00^{a(a)}$	$25.00\pm3.75^{b(b)}$	$50.00\pm1.75^{b(c)}$
0.0005	$3.35\pm1.00^{a(a)}$	$11.65\pm2.10^{ab(a)}$	$50.00\pm2.75^{c(b)}$	$70.00\pm3.15^{c(c)}$
0.001	$4.12\pm1.65^{a(a)}$	$13.65\pm1.31^{b(a)}$	$60.00\pm1.65^{cd(b)}$	$85.00\pm2.65^{d(c)}$
0.005	$6.00\pm1.13^{a(a)}$	$15.00\pm2.90^{b(a)}$	$65.00\pm0.75^{d(b)}$	$100.00 \pm0.00^{e(c)}$
0.01	$10.00\pm2.90^{ab(a)}$	$23.35\pm4.40^{bc(b)}$	$88.35\pm3.40^{e(c)}$	$100.00\pm0.00^{e(d)}$

Mean values followed by the same letter(s) are not significantly different (P>0.05) by Tukey's Test. Letters immediately following the means are for vertical comparison while letters in parenthesis are for horizontal comparison.

Table 5 . Effect of DDVP on the mortality (%mean ± S.E.) of *C. maculatus* population from Ibadan

	Duration (Hours)			
Concentration %	3	6	24	48
0.0	0.00±0.00$^{a(a)}$	0.00±0.00$^{a(a)}$	0.00±0.00$^{a(a)}$	0.00±0.00$^{a(a)}$
0.00001	0.00±0.00$^{a(a)}$	1.65±0.01$^{a(a)}$	20.00±2.90$^{b(b)}$	46.65±2.80$^{b(c)}$
0.0001	1.65±0.65$^{a(a)}$	5.00±1.90$^{a(a)}$	23.35±1.65$^{b(b)}$	50.00±5.75$^{b(c)}$
0.0005	3.35±0.65$^{a(a)}$	10.00±0.20$^{ab(a)}$	38.35±0.40$^{c(b)}$	53.35±2.40$^{b(c)}$
0.001	5.35±0.65$^{a(a)}$	16.65±1.05$^{bc(ab)}$	48.35±0.13$^{cd(c)}$	95.00±1.15$^{c(d)}$
0.005	11.65±0.20$^{a(a)}$	26.65±1.30$^{cd(b)}$	50.00±0.75$^{cd(c)}$	97.00 ±1.00$^{c(d)}$
0.01	13.65±1.40$^{ab(a)}$	30.15±1.40$^{d(b)}$	55.00±0.13$^{d(c)}$	100.00±0.00$^{c(d)}$

Mean values followed by the same letter(s) are not significantly different (P>0.05) by Tukey's Test. Letters immediately following the means are for vertical comparison while letters in parenthesis are for horizontal comparison.

Table 6. Effect of DDVP on the mortality (%mean ± S.E.) of *C. maculatus* population from Laboratory culture

	Duration (Hours)			
Concentration %	3	6	24	48
0.0	0.00 ± 0.00$^{a(a)}$	0.00 ± 0.00$^{a(a)}$	0.00 ± 0.00$^{a(a)}$	0.00 ± 0.00$^{a(a)}$
0.00001	0.00±0.00$^{a(a)}$	6.65±0.65$^{a(a)}$	30.00±2.65$^{b(b)}$	60.00±6.00$^{b(c)}$
0.0001	1.65±1.65$^{a(a)}$	9.65±1.12$^{ab(a)}$	41.65±2.00$^{bc(b)}$	61.65±5.75$^{b(c)}$
0.0005	11.65±0.33$^{ab(a)}$	16.65±1.65$^{b(a)}$	55.00±0.25$^{c(b)}$	90.00±5.75$^{c(c)}$
0.001	13.65±1.33$^{b(a)}$	21.65±0.40$^{bc(a)}$	73.35±0.65$^{d(b)}$	100.00±0.00$^{c(c)}$
0.005	14.35±0.00$^{bc(a)}$	24.65±1.15$^{bc(a)}$	78.35±0.25$^{d(b)}$	100.00 ±0.00$^{c(c)}$
0.01	16.35±0.35$^{bc(a)}$	27.00±0.65$^{c(ab)}$	88.35±0.40$^{e(c)}$	100.00±0.00$^{c(d)}$

Mean values followed by the same letter(s) are not significantly different (P>0.05) by Tukey's Test. Letters immediately following the means are for vertical comparison while letters in parenthesis are for horizontal comparison.

3.2. Lethal Concentrations of DDVP and Resistance Factors of C. maculatus Populations

The concentrations of DDVP required for achieving 75% (LC$_{75}$) and 95% (LC$_{95}$) mortality in the various populations of *C. maculatus* as well as each population's corresponding resistance factors after 24 hours post-treatment are shown in Table 7. Bruchids, obtained from Ibadan, required the highest concentration (LC$_{75}$: 0.316 mg ml^{-1}; LC$_{95}$: 185.418 mg ml^{-1}) of DDVP while their counterpart from Ijan-Ekiti required the lowest concentration of DDVP (LC$_{75}$: 0.006 mg ml^{-1}; LC$_{95}$: 0.242 mg ml^{-1}). Similarly, lethal concentration (LC$_{75}$ and LC$_{95}$) values of bruchid population obtained from Ibadan were significantly higher ($P < 0.05$) than those obtained from Akure, Ikere-Ekiti and laboratory culture, respectively, as inferred from their fiducial limit values. Of all the populations sampled, the highest Resistance Factor (RF) was observed in bruchids population obtained from Ibadan (RF$_{75}$:158.00; RF$_{95}$: 1483.30), while the lowest RF was observed in those obtained from Ijan-Ekiti (RF$_{75}$:3.00; RF$_{95}$: 1.94).

Table 7. Lethal concentrations (LC$_{75}$ and LC$_{95}$) (mg ml^{-1}) of DDVP and resistant factor of *C. maculatus* populations at 24 hour post-treatment

Location	Slope (±S.E)	LC$_{75}$	RF$_{75}$	LC$_{95}$	RF$_{95}$
Akure	0.56 (±0.06)	0.007 (0.004-0.016)	3.50	0.411 (0.128-2.256)	3.29
Ikare-Akoko	0.43 (±0.05)	0.022 (0.009-0.085)	11.00	3.963 (0.644-76.546)	31.70
Ikere-Ekiti	0.65 (±0.06)	0.010 (0.006-0.022)	5.00	0.328 (0.120-1.364)	2.62
Ijan-Ekiti	0.61 (±0.06)	0.006 (0.002-0.126)	3.00	0.242 (0.025-214.093)	1.94
Ibadan	0.35 (±0.06)	0.316 (0.066-5.329)	158.00	185.418 (9.308-48677.02)	1483.30
Laboratory	0.56 (±0.06)	0.002 (0.001-0.004)	1.00	0.125 (0.045-0.543)	1.00

SE: Standard error; LC: Lethal concentration; RF: Resistance factor. Values in parenthesis represents 95% Fiducial limits.

4. Discussion

In the present study, the response of various Nigerian populations and laboratory culture of *C. maculatus* to DDVP was evaluated. The results obtained indicated that the mortality of bruchid samples from each location varied with different concentrations of insecticide and exposure time. Except for the Laboratory (reference) population, less than 50% mortality was observed in all the locations with the lowest experimental concentration even at the highest duration (48 hours). *C. maculatus* is known to mate shortly after emergence, with the majority of eggs laid within three days (Fox, 1993; Ofuya, 1995). Hence, bruchid populations, in the present study, would have mated and laid egg before being killed. Although DDVP is known to be active against immature stages of stored product insects within grains (Semple et al., 1992), eggs already laid by the adult bruchid usually lead to a loss in the aesthetic and the marketability value of the infested cowpea seeds (Swella and Mushobozy, 2007). This might be responsible for post-harvest losses usually incurred on stored cowpea seeds in Nigeria despite the use of synthetic insecticides in most cases (Singh and Ntare, 1985; Baidoo et al., 2010).

Insecticide resistance refers to the insecticide selected inheritable ability of insects' population to withstand the exposure to a dose of an insecticide that would kill the majority of a normal (susceptible) population of the same species (Buhler, 2013). Lethal concentrations and resistance factors of the sampled populations revealed that Ibadan population of *C. maculatus* showed the highest resistance to DDVP. High resistance of the bruchid sample from Ibadan may be linked to the strategic location of this city, being in Southern-Western part of Nigeria. Ibadan is the largest city in West Africa and the second largest in all Africa (Kumassah, 2009). It is also the third cheapest Nigerian city to live in and it contains a large human population (about 2.949 million as at 2011) (Ejiofor, 2014; NDP, 2014). There are several markets and storage facilities within and around the metropolis. Hence, more insecticides might have been used in the management of diverse stored product pests in most stores. For instance, in Nigeria, DDVP is one of the most common insecticides used directly on cowpea seeds before being bagged for storage. The repeated exposure of bruchids to this insecticide overtime might have led to their possible resistance to this insecticide. On the contrary, the low resistant factor of the bruchid population from Ijan-Ekiti to DDVP suggests that this insecticide might not have been over-used on bruchids sampled from this town which is the smallest town of all the locations sampled. Fragoso et al. (2002), Pereira et al. (2006) and Odeyemi et al. (2010) had earlier ascribed the variation in the resistance of insect from different locations to the greater use of insecticides and to the usage pattern in those locations.

The differences in the resistance factor between insect populations to a particular insecticide have been attributed to several factors. Such factors include: thickness of insect's exoskeleton, type of insecticide being used, the ability of the insect to metabolize a poison, concentration of insecticide used, time of exposure, the type of food eaten by the insect, insect location and species, among other parameters (Gbaye et al., 2011; Buhler, 2013; Oyeniyi et al., 2015a; Oyeniyi et al., 2015b). Some of these factors might have contributed to the variations observed in the resistance factor of Bruchid populations sampled in the present study. Variations in the resistance status of Bruchid populations observed in the presentstudy is in line with the various reports on the resistance of diverse insect pests to synthetic insecticides (Jermannaud, 1994; Perez et al., 2000; Pereira et al., 2006).

A concentration of DDVP higher than 0.01% (0.01mg ml-1 which is the maximum residue limit (MRL) permitted by EU) would be required for effectiveness within 24 hours. However, due to various adverse effects associated with pesticide usage as well as a recent ban imposed on Nigeria by European Union owing to high level of DDVP (0.03-4.60 mg kg-1) in exported cowpea grains (Nigeria Punch of 30et July, 2015), increasing the concentration above 0.01% cannot be encouraged. Thus, to reduce the huge post-harvest losses, usually incurred due to inability of insecticides to ensure maximum protection of cowpea, there is a dire need to constantly monitor and manage the resistance to DDVP. In areas where resistance is observed, such as Ibadan, there is a need to discontinue the use of DDVP and replace it with other insecticides that have a different chemistry and mode of action. This is required while the search for sustainable alternative control measures to synthetic chemicals is on, especially under a large scale storage where botanical use is not realistic.

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Gastroprotective Activity of *Eruca sativa* Leaf Extract on Ethanol-Induced Gastric Mucosal Injury in *Rattus norvegicus*

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Abstract

Eruca sativa (Es), known as *jarjeer*, have been used in traditional medicine for the treatment of different diseases. First, its powder was subjected to Energy-Dispersive x-Ray Fluorescence Analysis to determine the mineral content. Then the plant was extracted by 95% of ethanol to evaluate anti-ulcerogenic activity against ethanol induced gastric ulcer. For this purpose, thirty rats were divided into 6 groups n=5. Respectively, all the animals were orally pre-treated with water, 10% Tween 20, omeprazole 20 mg/kg, 250, 500 and 750 mg/kg plant extract one hour before treating with absolute ethanol to generate gastric mucosal injury. After additional hour, rats were anaesthetized and sacrificed; the gastric content was then collected and stomachs were examined to determine mucosal lesions. The results showed that Es contains several beneficial minerals in which potassium showed to be in highest content 22.02±1.2%. Grossly and histologically, the Tween 20 group exhibited severe mucosal injury, whereas the pre-treated rats with plant extract exhibited a significant protection in dose dependent manner. Further, Es caused elevation of pH of gastric content and mucus production. Therefore, it can be concluded that Es-ethanol leaf extract exhibits an anti-ulcer activity against ethanol-induction model through maintaining the acid base balance of gastric content.

Keywords: *Eruca sativa*, Gastric ulcer, Anti-ulcer,Mineral content.

1. Introduction

Gastric ulcer is among the most serious and chronic diseases in the world. It is widely distributed among the world's population, affecting about 10% of them. It usually occurs in the stomach and near the duodenum (Abdulla et al., 2009). Now, it has been understood that gastric ulcer occurs when there is an imbalance between acid and pepsin together with the protective barrier present in the digestive tract (Mizui et al., 1987; Shaker et al., 2010). Many factors contribute to the etiology of the gastric ulcer. The following causes can significantly decline the defenses of the mucosal barrier of the stomach, which, in turn, raises the probability of getting an ulcer and slows the healing of the existing ulcers. These factors include intake of aspirin, nonsteroidal anti-inflammatory drugs (such as ibuprofen and naproxen), alcohol, stress or emotional, caffeine, cigarette smoking and radiation therapy (Hor et al., 2011).

Although the introduction of proton-pump inhibitors to the classic anti-ulcer therapy revolutionized the treatment of peptic ulcers and other gastrointestinal disorders, there is still no complete cure for this disease. Further, it has been shown that the long-term use of these drugs leads to various adverse and side effects; relapses of the malady, ineffectiveness of different drug regimens and even resistance to drugs are emerging(Al Mofleh et al., 2007).

Nowadays, following the traditional belief, the demand for herbal plants is increasing in the developing countries (Wasman et al., 2011). Traditionally, several plants have been used to treat a variety of diseases, including gastric ulcers.

Iraqi plants have been widely used because of their relevant aromas and tastes that add variety and flavor to foodstuffs. In Erbil- Kurdistan region, many of these plants are used to treat different human diseases but there is no phyto-therapeutic evidence (Naqishbandi, 2014). Here, among Kurdish and Arabian people, Es leaf is widely used in salads. Further, Greek medicine used Es leaf in diuretic, stimulant, and in the treatment of stomach disorders and scurvy (Alqasoumi et al., 2009). The seeds and the tender leaves are known in Arabian countries to increase the sexual desire and are considered to be an aphrodisiac (Alam et al., 2007). They are also used as a carminative and to alleviate abdominal discomfort and to improve digestion. Therefore, in the present study, Es leaf

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has been selected for evaluating anti-ulcer ability in laboratory rats.

2. Materials and Methods

2.1. Drug (Omeprazole)

In the present study, omeprazole (OMP) (Charcop, Kandivli, India) was used as a standard positive anti-ulcer drug; it was obtained from a local Pharmacy in Erbil city-Iraq. The drug was dissolved in10% Tween 20 and administered orally to the rats in a concentration of 20 mg/kg body (5 ml/kg) (Wasman et al., 2011).

2.2. Plant material and preparation of extract

Fresh *Eruca sativa* leaves were purchased from a local vegetable market in Erbil, and the identity of these leaves were confirmed by Dr. Abdullah Shakor, a taxonomist from the Department of Biology College of Education University of Salahaddin-Erbil-Iraq. After identifying Es, the leaf parts of the plant was cleaned, dried in the shaded place for 7-10 days and they were finely powdered using electrical blender; then they were stored in dark glass flasks to protect them from light and molds. An amount of 100g of coarsely pulverized Es leaves were placed in a glass percolator with 1000ml of 95% ethanol and were allowed to stand at room temperature for about 72 h (Wasman et al., 2011). After 3 days, the mixture was filtered using a fine muslin cloth followed by filter paper (Whatman No. 1) and distilled under a reduced pressure in a rotary evaporator (RE 200B/UK) (Donald et al.,1982). The *Eruca sativa* ethanolic leaf extract (ESELE) was then dissolved in Tween 20 (10% v/v) and administered orally to rats in concentrations of different doses (Rouhollahi et al., 2014).

2.3. Determination of Mineral

Energy-Dispersive X-Ray Fluorescence Analyser (CIT-3000 MP)(Sichuan, China) was used to determine the mineral content of Es. For this reason, the amount of 100gm of Es dried powder was weighed and subjected to the (CIT-3000 MP); the amount of each element represents the quantity in 100gm of plant material. This experiment was applied in triple and then the mean was calculated (Bozokalfa et al., 2011).

2.4. Experimental Animals

Healthy thirty adult male rats were obtained from the Experimental Animal House, College of Medicine/Haweler Medical University. The animals were kept at room temperature in humidity rooms on a standard light/dark cycle (12 h light; 12 h dark cycle) at (22±3C°). Rats weighing between 150g - 200g were placed individually in separate plastic cages (56 x 39 x 19), bedded with wooden chips in the animal house of Biology Department /College of Education /Salahadin University-Erbil. The rats were fed with standard rat diet chow and tap water. They were kept under observation for about two weeks before the initiation of the experiment. All the procedures described were reviewed and approved by the Institutional Animal Ethical Committee. Throughout the experiments, all animals received human care according to the criteria outlined in the “Guide for the Care and Use of laboratory Animals,” prepared by the National Academy of Sciences and published by the national Institute of health.

2.5. Antiulcer Experiment

The ethanol ulcer induction experiment of the present study was adopted depending on the method described in previous studies (Garg et al., 1993; Mahmood et al., 2010).

Rattus norvegicus rats (150–200 g) were deprived of food for 48 h before the experiment was conducted in order for the stomach to be empty, but they were allowed free access to drink water up until 2 h before starting the experiment (Mahmood et al., 2010). All rats were treated by orogastric intubations. The animals were divided randomly into six groups, each consisting of five rats; they were treated as shown in Table1.

Table 1. Design of antiulcer experiments

Groups	Treatments	Concentrations
Group(1) Normal	Water	-
Group(2) (negative control group)	Tween 20 10% v/v,	5 mL/kg
Group(3) (positive control group)	Omeprazole 20 mg/kg,	5 mL/kg
Group(4) first dose	ESELE 250 mg/kg,	5 ml/kg
Group(5) second dose	ESELE500mg/kg,	5 ml/kg
Group(6) third dose	ESELE750mg/kg,	5 ml/kg

2.5.1. Gastric Ulcer-Induction by Ethanol and Tissue Sample Collection

The rats were starved for 48 h before the experiment, but they were allowed free access to drinking water up till 2 h before the experiment. Gastric ulcer in *Rattus norvegicus* was induced by orogastric intubation of absolute ethanol (5 ml/kg) (Alrdahe *et al*., 2010). All animals were anaesthetized by intraperitonial injection with ketamine (100 mg/ml) and xylazine (100mg/ml) in a ratio 4:1 (v/v) (HIKMA pharmaceuticals, Amman-Jordan). The animals were sacrificed and their stomachs were tied from up and down to preserve the gastric juice for measuring the gastric acid; then the stomachs were excited and fixed in formalin 10% for histological examination.

2.5.2. Measurement of Acid in Gastric Juice

Each stomach was opened along the greater curvature. Samples of gastric contents were analyzed for hydrogen ion concentration by pH-meter using 3540 pH Conductivity Meter (JENWAY-Japan).

2.5.3. Measurement of Mucus Production

The gastric mucus production was measured in all the experimental rats that were subjected to absolute ethanol-induced gastric mucosal injury. The gastric mucosa of each rat was gently scraped using a glass slide and the mucus obtained was weighed using a precision electronic balance (Tan *et al*., 2002).

2.5.4. Histological Preparation

A histological examination was performed after the assessment of ulcer lesion. The stomachs were fixed in 10% of buffered formalin solution. Tissue processing (dehydration, cleaning and infiltration) was done automatically using automated tissue processor (Leica TP1020). Then, the tissues were embedded in paraffin wax using Leedo HISTOEMBEDDER. The embedded tissues were sectioned with microtome to produce 5 µm paraffin wax tissue sections. Then, the sections were stained with haematoxylin and eosin followed by mounting with DPX mounting media. Next, the mounted sections were evaluated for microscopic examination using light microscope (AmScoop microscope eyepiece camera. China).

2.6. Statistical Analysis

All data were analyzed by Statistical Package Social Science (SPSS) version 17.0. One-way ANOVA is used to show the mean differences between all samples (* $p \leq 0.05$).

3. Results

3.1. Determination of Mineral

In this experiment, different minerals have been detected in *Eruca sativa* leaf powder using Energy-Dispersive X-Ray Fluorescence Analyser, as shown in Table (2). The results show that potassium, sulphur and calcium are in large quantity while cerium, iodine, phosphorus, bismuth and selenium are relatively low compared to the other elements. Interestingly, the potassium was detected in a large percentage: 22.02±1.2%**.**

Table 2. Mineral composition of *E. sativa* leaf powder

Plant	Eruca sativa	Leaf
Minerals (%)	Calcium Ca	5.23± 0.9
	Potassium K	22.02±1.2
	Sulfur S	6.58±0.7
	Cerium Ce	0.0006±0.1
	Iodine I	0.001±0.3
	Phosphorus P	0.0017±0.4
	Bismuth BI	0.0036±0.3
	Selenium Se	0.0024±0.6

This experiment has been applied in triple and then the mean±SE was calculated

3.2. Gross Evaluation of Gastric Lesions

Results showed that the rats pre-treated with *Eruca sativa* ethanolic leaves extract (ESELE)had significantly reduced the areas of gastric ulcer formation compared to the rats pre-treated with only 10% Tween 20 (ulcer control group). As shown in Figure 1, the rats pre-treated with 10% Tween 20 showed severe damage and injuries of gastric mucosa, as shown in Figure (1B). The rats pre-treated with plant extract significantly suppressed the formation of the mucosal injuries but some folds were still noticed in the rats pre-treated with 250 and 500 mg/kg (Figure 1D&E). On the other hand, for the rats pre-treated with750 mg/kg of ESELE, a complete protection of gastric mucosa was observed with the flattening of gastric mucous wall as in (Figure 1F).

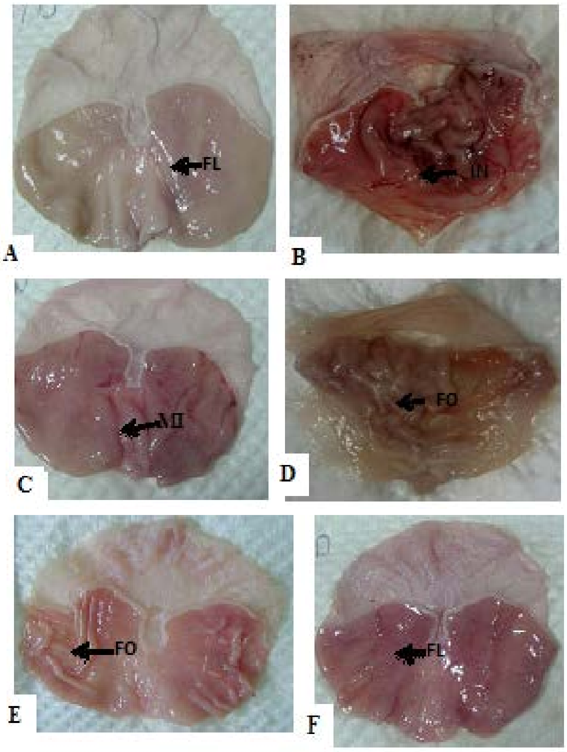

Figure 1. Macroscopic appearance of the gastric mucosa in rats. **A,** no treated with any material (Normal).**B,** Pre-treated with 5 ml/kg of 10%Tween 20 (ulcer control). Severe injuries (IN) were seen in the gastric mucosa. **C,** pre-treated with 5 ml/kg of Omeprazole (20 mg/kg). Injuries to the gastric mucosa were milder (MI) compared to the injuries seen in the ulcer control rat. **D,** Pre-treated with 5 ml/kg of ESELE (250 mg/kg). Mild injuries with folded of gastric mucosa were seen (FO)**. E,** Pre-treated with 5 ml/kg of ESELE (500 mg/kg). No injuries with folded of gastric mucosa were seen (FO).**F,** pre-treated with 5 ml/kg of ESELE (750 mg/kg). Protection of gastric mucosa was more prominent and the flattening of gastric mucosa were seen (FL).

3.3. pH of Gastric Content and Mucus Production

The effect of ESELE on gastric acidity and mucus production in the ethanol-induced gastric lesion model is shown in Table (3). The acidity of gastric content significantly ($P \leq 0.05$) decreased in experimental animals pre-treated with 500 and 750 mg/kg of ESELE and the omeprazole group compared with that of the ulcer control group. While rats pre-treated with 250 mg/kg of ESELE did not show any effect in the pH level of gastric juice and mucus production, as shown in the Table 3, that there is no significant differences ($P \leq 0.05$) compared with the negative control group. The mucus production of the gastric mucosa significantly increased ($P \leq 0.05$) in animals pre-treated with ESELE in rats pre-treated with 500 and 750 mg/kg and omeprazole compared with the ulcer control group as in (Table 3).On the other hand, there were no significant differences in the mucus content between omeprazole and 500 and 750 mg/kg ESELE groups. Rats pre-treated with 250 mg/kg of ESELE did not show any effect in the pH level of gastric juice and mucus production, as shown in Table 3, that there are no significant differences (P≤ 0.05) compared with the negative control group.

Table 3. Effect of ESELE on pH of gastric content and mucus in rats.

Animal Group	Pre-treatment (5 ml/kg dose)	pH of gastric content	Mucus content
1	Normal	6.68 ±0.2*	0.67±1.1*
2	10% Tween 20(Ulcer control)	3.6 ± 0.10**	0.31±0.8
3	Omeprazole (20 mg/kg) (positive control)	6.84 ± 0.17*	0.62±0.9*
4	LD ESELE (250 mg/kg)	4.3 ± 0.23**	0.37±1.9
5	MD ESELE (500 mg/kg)	6.9 ± 0.20*	0.55±0.5*
6	HD ESELE (750 mg/kg)	7.2 ± 0.4*	0.60±1.7*

All data expressed in mean ± SEM. * Significant difference (p≤0.05) with 10% Tween 20 (Ulcer control),** Significant difference (p≤0.05) with omeprazole (positive control).

3.4. Histological Evaluation of Gastric Lesions

Histological observation of ethanol induced gastric lesions in ulcer negative control group, pre-treated with only 10% Tween 20, showed a comparatively extensive damage to the gastric mucosa, edema and leucocytes infiltration of the submucosal layer (Figure 2 B).

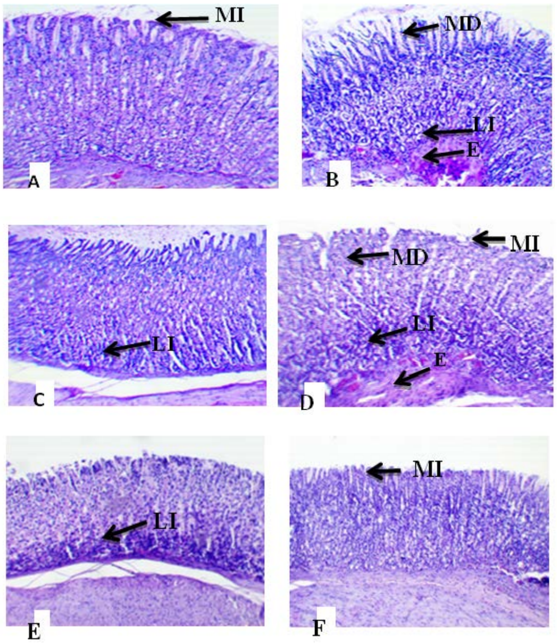

Figure 2. Histological section of gastric mucosa in a rat. A, treated with water as normal. It shows normal surface epithelium (Mucosal intact) and normal submucosal (H&E stain 100X).B, pre-treated with 5 ml/kg of 10%Tween 20 (ulcer control). There was severe disruption to the surface epithelium or mucosal damage (MD), and edema (E) of the submucosa layer with leucocytes infiltration (LI) (H&E stain 100X).C, pre-treated with 5 ml/kg of Omeprazole (20 mg/kg). It shows leucocytes infiltration (LI) D, pre-treated with 5 ml/kg of ESELE (250 mg/kg). (E) pre-treated with 5 ml/kg of ESELE (500 mg/kg).F, pre-treated with 5 ml/kg of ESELE (750 mg/kg).It shows mucosal intact (MI) and normal submucosa.

Rats pre-treated with ESELE had a comparatively better protection of the gastric mucosa as proven by the reduction in the ulcer area, reduced or absence of submucosal edema and leucocytes infiltration (Figure 2 D, E&F). The ESELE was shown to exert the cytoprotective effects in a dose-dependent manner.

4. Discussion

Normally, there is a balance between the protective factors (e.g., mucus, bicarbonate, prostaglandins, nitric oxide and normal blood flow) and aggressive factors (e.g., acid plus pepsin, active oxidants, leukotrienes, endothelins, bile or exogenous factors including non-steroidal anti-inflammatory drugs and ethanol). Gastric ulcer develops when the aggressive factors overcome the protective mechanisms (Borrelli and Izzo, 2000).

It is known that gastric lesions, produced by ethanol administration, appear as multiple-hemorrhagic red bands of different size along the glandular stomach. Absolute ethanol is commonly used for inducing ulcer in experimental rats and lead to intense gastric mucosal damage (Abdulla *et al*., 2010). Studies suggest that the ethanol damage to the gastrointestinal mucosa starts with microvascular injury, namely disruption of the vascular endothelium resulting in increased vascular permeability, edema formation and epithelial lifting (Szabo *et al*., 1995).Ethanol produces necrotic lesions in the gastric mucosa by its direct toxic effect, reducing the secretion of bicarbonates and production of mucus (Marhuenda *et al*., 1993). The exposure to ethanol increases the extension of the cellular damage in a dose-dependent way (Mutoh *et al*., 1990).

In the present study, we observed a flattening in the mucosal wall, which suggests that the anti-ulcer effect of ESELE might be due to a decrease in the gastric motility. It is reported that the changes in the gastric motility may play a role in the development and prevention of experimental gastric lesions (Garrick *et al*., 1986; Takeuchi *et al*., 1988; Abdulla *et al*., 2010). The relaxation of the circular muscles may protect the gastric mucosa through flattening the folds. This increases the mucosal area exposed to necrotizing agents and reduce the volume of the gastric irritants on rugal crest (Takeuchi and Nobuhara, 1985). Rats treated with ESELE (250mg/kg, 500mg/kg and 750mg/kg) and those treated with omeprazole displayed a better protection of their gastric mucosa as seen by the reduction of the ulcerated areas. The reduced submucosal edema and the inflammatory reactions were also observed in these groups.

The results of this study showed that the ESELE possesses significant anti-secretory, anti-ulcer and cytoprotective properties in rats. Pre-treatment with ESELE produced a dose-dependent decrease in the gastric acidity and an increase in mucus content. The anti-ulcerogenic activity of the extract was also confirmed histologically. Histology studies confirmed the efficacy of ESELE supplementation in preventing ethanol-induce hemorrhage and necrosis in the superficial layer of the gastric mucosa. The cytoprotective effect of the extracts could be partially due to their flavonoid content and to their reactive oxygen species scavenging property (Sanchez *et al*., 2001).

Since ESELE markedly inhibited a gastric acid secretion and ruminal ulcers in ethanol induced rats, this observed effect could be related, at least in part, to the ability of ESELE to reduce gastric acid secretion. It is now accepted that the gastric acid secretion plays an important role in the progression from an erosive mucus layer to a gastric lesion. On the other hand, substances, which have the ability to suppress gastric acid secretion, such as proton pump inhibitors (Omeprazole) and histamine H2-receptor antagonists, are believed to accelerate the healing process of the gastric lesions or inhibit the formation of mucosal injury(Brzozowski *et al*.,2000).

The preliminary phytochemical screening of Es revealed the presence of flavonoids, sterols and/or triterpenes. Moreover, quercetin and its derivatives were also reported in Es leaves. Previous studies have shown that flavonoids may be related to the anti-ulcer activity (Hiruma-Lima *et al*., 2006), and play a major role in the mechanism of gastro-protection through the rising pH of gastric juice (Havsteen, 2002; La Casa *et al*., 2000).

Elements play a crucial role in the medicinal value of a plant, in health and in curing disease. They play a nutritive, catalytic and balancing function in plants. Plants take them from the ground and incorporate them into organic compounds that we consume through eating either the plants or the animals that eat them (Joyo *et al*., 1997). In the present study, K^+, S and Ca^{++}were detected in a large quantity while Ce, I, P, Bi and Se are relatively low compared to the other elements. The K^+ is found in a large percentage (22.02%). Our findings are in accordance with the study of Bozokalfa *et al*. (2011) who showed that the leaves of *Eruca sativa* contain a large amount of important mineral elements for human nutrition, particularly K^+, Ca^{++} and P concentrations. Due to the deficiency of these minerals in human diet, most of these minerals are often taken as supplements for their important role in human health (Agarwal *et al*., 2011). Some of these elements are directly related to the anti-ulcer ability, as it was reported previously by Kim *et al*. (2012), who demonstrated that selenium inhibits the formation of ethanol-induced gastric mucosal lesions through the prevention of lipid peroxidation and the activation of enzymatic radical scavenging. Chai (2011) also reported that compounds that contain bismuth are often used in the three-drug treatment programs of gastric ulcer; they destroy the cell walls of *Helicobacter pylori* bacteria.

5. Conclusion

In conclusion, *Eruca sativa* leaf extracts could significantly protect the gastric mucosa against ethanol-induced injury. Such protection was shown to be dose-dependent as ascertained by the reduction of the ulcer areas in the gastric wall as well as by the reduction or the inhibition of edema and the leucocytes infiltration of sub-mucosal layers. Particularly at a dose of 750 mg/kg leaf extract, this protection could be due to the balance between acid-base production in stomach and the mineral content of the plant itself.

Further studies are required to determine the phytochemical compounds responsible for the mechanism of antiulcer of *Eruca sativa* leaf extracts.

Acknowledgment

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Spatial-Temporal Variation in Algal Community in Freshwater Springs Inhabited by Aquatic Salamander *Neurergus crocatus*

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Abstract

The basic idea of the present study was to assess the population of algal flora in two different aquatic ecosystems inhabited by a salamander *Neurergus crocatus.* According to the results of physico-chemical parameters, there were significant differences between the two ecosystems in all the measured parameters, and the two locations characterized by low nutrient contents. At site one, the variation in all physico-chemical parameters was significant and there was no stability of the water quality parameters during the period of study, while site two was characterized by a sort of stability in all physico-chemical parameters of water quality. Regarding the algal flora at two sites, there was a clear variation in this aspect. At site one, three species of macrobenthic and seven species of microbenthic algae were identified during the period of study. Whereas in site two, only one species of red algae and one species of cyanobacteria was noticed in March. Low number of algal species in this site can be attributed to the lytic activity of *Pseudomonas fluorescens*.

Keywords: : macrobenthic, microbenthic, salamander, *Pseudomonas fluorescens*.

1. Introduction

Over two thirds of the Earth’s surface are covered by water and less than a third is taken up by land. As Kurdistan population continues to grow, people are putting pressure on the Kurdistan’s water resources. Rivers, springs, lakes and other inland waters are being affected by the human activities. Water resources in Kurdistan are productive water, and they provide drinking water to many villages; but all these resources are being threatened by human activity. During the last ten years, people widely went out for pleasure and recreation leaving huge quantities of their litter and residue at the watershed of water recourses, which led to the environmental disturbance changes in the structure and function of biological systems. Ecological assessment of the water body includes both chemical and biological indicators of water quality (Zhang, 2006). Algae are one of the biological indicators used for the measurement of water quality (Allison *et al.*, 2014) and constitute an important component of wetland and springs (Robinson *et al.*, 2000). Springs usually lack true phytoplankton, but may have benthic algae because spring waters are shallow and have abundant submersed substrata utilized for colonization (Sanley *et al*., 2003). The algal distribution pattern in waters usually indicates the type of environment they inhabited; therefore algae have been widely used as an ecological indicator. Algae are naturally found in all types of ecosystems and can indicate the conditions of an ecosystem, so the presence of a certain species can indicate the amount and type of the available nutrients (Whitton, 1979; Symoens *et al*., 1981; Hosmani and Bharati, 1982; Austin and Deniseger, 1985). *Neurergus crocatus*an aquatic salamander but it is not commonly found in aquatic habitats in Kurdistan region. It is well known that in an ecosystem when chemical, physical and nutritional requirements of a specific organism are not provided it cannot survive (Thomas and Smith, 2012). The problem of pollution is widely spread in Kurdistan Region. Therefore, studying the algal flora of aquatic ecosystem inhabited by the salamander *Neurergus crocatus* will indicate its physical, chemical and biological requirements. In the present study, we reviewed the recent publications in combination with classical freshwater approaches to highlight the importance of freshwater benthic algal ecology. So, the aims of this study are:

1. To study the spatial and temporal variation in algal community in two aquatic ecosystems inhabited by the salamander *Neurergus crocatus*.
2. To describe spatial and temporal differences patterns in the physical and chemical conditions of the two studied aquatic ecosystem environments.
3. To diagnose which alga is a bio indicator for such aquatic habitat.

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2. Materials and Methods

2.1. Field Sampling

Two aquatic habitats inhabited by *Neurergus crocatus* were selected at Duhok province. The first site is located 40 km north east of Duhok city near Swaratoka resort in a deep canyon surrounded by a rugged mountain, and its freshwater creek emanates from a spring at the foot of the Gare Mountain (Plate 1) located at 37º 1′ 29″ N and 43º 15′ 31″ E. The second site is a freshwater pool, located 70 km far away from the first site near Sheladezi village at 39º 17′ 25″ N and 45º 37′ 8″ E, its water originates from a spring at the foot of Gare Mountain. The bottom of the pool is covered with calcareous stones (Plate 2). The first site is located at a region with a low human disturbance at the watershed, while the second site is commonly used as a public place for local picnics.

Plate 1: View of site one

Plate 2: View of site two.

2.2. Sample Collection and Analysis

The present study was conducted for five months, from January to May 2014.Water and algal samples were collected monthly. Water samples were taken randomly from the aquatic habitat by plastic bucket, while epipelic, epilithic, and epiphytic algae were collected from different places of each location and preserved in a glass container for identification. The parameters of water quality, which were measured in the field, included: air and water temperatures using mercury thermometer, dissolve Oxygen using portable DO-meter (Model 407510, EXTECH Instruments), pH using portable pH-meter (Model 430, JENWAY), and electric conductivity using portable conductivity-meter (Model inoLab Cond level 1 E 163694).

Total hardness, total alkalinity, calcium hardness, magnesium hardness, nitrate and phosphate were measured according to A.P.H.A. (1998).Three replicates of water sample from each parameter were analyzed at the Department of Biology, Faculty of Science, University of Duhok. Soft algae were identified under magnification (40 X) of light microscope. Cleaning of diatoms was carried out according to Patric and Riemer, (1966) and fixed on slides for identification. Identification and classification of macrobenthose and microbenthose were done to species level according to the methods described by Desikachary (1959), Patrick and Riemer (1966), Weber (1971), Prescott (1975), Edward and David (2010). Yellow-greenish stones, collected from site two, grossly expected to be algae but when examined microscopically no algae were found and only bacterial species were dominated. For bacterial identification, samples were cultured on blood agar (Oxoid, UK), McConkey agar (Oxoid, UK) and Nutrient agar (Oxoid, UK). All inoculated culture media were incubated at 37 ºC under aerobic conditions for 24 hours. Preliminary tests such as Gram stain, oxidase test, triple sugar iron agar and motility tests were used.

2.3. Statistical Analysis

Data were analyzed statistically using excel and GraphPad Prism 5 using XY analysis (nonlinear regression) by plotting concentration of parameters on Y axis and periods of parameters on X axis followed by Tukey's test for comparing each parameter's concentration in different periods. *P*-values < 0.05 were considered statistically significant. Common letters between any two mean values express no significant difference.

3. Results and Discussion

Abiotic factors

The average values of the selected parameters in each location were measured during the study period as shown in Table 1 and Figure 1. The results of the air temperature at site one ranged from 6 to 23ºC, while at site two it ranged from 7.5 to 25ºC. Based on the results, there was a significant temporal variation in the air temperature in each site during the period of study, while a significant spatial variation in air temperature was found in March and May. The temporal variation in the air temperature depends on the climate of the region which is close to the Irano-turanian type (Guest, 1966). A significant spatial variation in the air temperature is due to the time of sampling and the weather conditions.

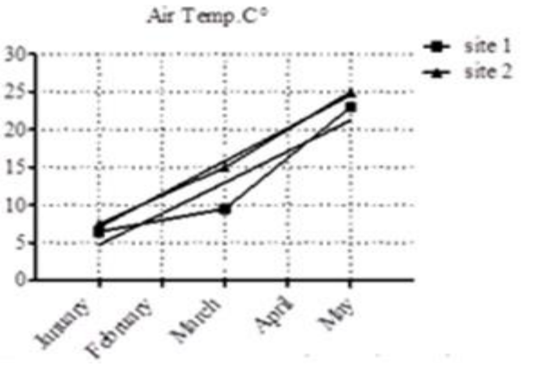


Figure 1. Air temperature at the two sites during the period of study

Table 1. Values of physico-chemical parameters of the water at the two sites during the period of study. Note: Small letters express statistical differences between different months in the same location while capital letters express statistical differences between the same months in different locations at *p*=0.05.

Months	Site 1			Site 2		
variables	January,2014	March	May	January	March	May
Air tem. Cº	6.5±0.115aA	9.5±0.057bA	23±0.057cA	7.5±0.057aA	15±0.577bB	25±0.577cB
Water Tem. Cº	4.9±0.057aA	7.7±0.057bA	16±0.577cA	12.7±0.0577aB	12.6±0.1000aB	13±0.5774aB
Ecµs/cm^2	1516±3.055aA	1272±2.082bA	1084±3.512cA	690±2.517aB	690±2.082aB	672±1.528bB
TDS mg/l	1364±4.163aA	1145±1.528bA	975±3.0cA	621±1.732aB	621±1.528aB	605±1.155bB
pH	8.08±0.0116aA	8.3±0.0577bA	8.33±0.0116bA	7.5±0.0577aB	7.21±0.0116bB	7.29±0.0116bB
DO(mg/l)	7±0.1528aA	6.7±0.100aA	6.5±0.1528aA	4.5±0.0577aB	4.1±0.0577bB	3.8±0.0577cB
NO_3^-µg/l	191.3±0.173aA	104.2±0.152bA	114.7±0.173cA	335.4±0.288aB	212.8±0.321bB	239.3±0.153cB
PO_4^{--}µg/l	97.8±0.208aA	30±1.0bA	44.2±0.145cA	84.43±0.0929aB	28±1.0bB	42.1±0.115cB
TH mg CaCo$_3$/l	552±3.055aA	490±3.055bA	460±2.082cA	289±2.309aB	291.5±0.252aB	271±1.528bB
Ca H(mg/l)	260±1.732aA	238±1.528bA	195±2.082cA	203±2.517aB	212.5±0.3055cB	175±1.0bB
Mg H(mg/l)	292±1.528aA	252±2.646bA	265±1.000cA	86±1.0aB	79±0.2333aB	96±2.517bB
Ca^{++}mg/l	104.2±0.7229aA	95.4±0.611bA	78.16±0.829cA	81.36±1.01aB	85.17±0.122bB	70.14±0.4000cB
Mg $^{++}$mg/l	70.96±0.3688aA	61.24±0.6413bA	64.4±0.2433cA	20.9±0.2433aB	19.2±0.05859aB	23.33±0.6106bB
Alkalinity mg CaCo$_3$/l	260±1.732aA	255±1.528aA	226±1.528bA	197±1.155aB	190±1.528aB	156±2.082bB

Water temperature in site one varied from 4.9 to 16 ºC, while in site two it varied from 12.6 to 13 ºC. The temporal variation in water temperature at site one was 11.1ºC. Water temperature at site one showed a significant variation in the different months of the study (Table 1).This wide range of water temperature can be attributed to the shallow depth of water in this site which can be easily affected by seasonal changes (Hassan *et al.*, 2008). In site two, a very narrow variation in the water temperature (0.4ºC) was noticed (Figure 2) which was not significant at the different times of sampling.

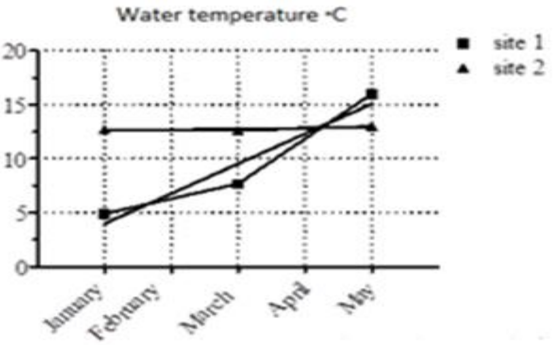


Figure 2. Water samples temperatures at the two sites during the period of the study

This result shows the steady state environmental condition of the aquatic ecosystem of freshwater spring which, within temperature, aeration, and nutrient supply, remains relatively constant throughout the year (Allan and Castillo, 2007; Aloisie *et al.*, 2008). There was a significant spatial variation in the water temperature between the two sites during the period of the study.

At site one, the EC ranged from 1084 to 1516 µs/cm, while at site two it ranged from 672 to 690 µs/cm. At both sites, the lowest value was measured in May, whereas the highest value was measured in January. It is clear from the results of EC (Figure 3) that the temporal variation at site one was 432 µs/cm, and there was a significant variation in the results of EC between the periods of the sampling. This wide range of variation in EC is related to the climate and the season of the sampling. The highest value of EC in January was due tothe rainy and snowy conditions which cause the dissolution of Ca and Mg salts from the rocks (Onyema and Emmsnuel, 2009; Barinova and Tavassi, 2009).The temporal variation in EC in site two was 18 µs/cm and this narrow variation can be attributed to the underground water which is not affected by the climate (Aloisie *et al.*, 2008). Statistically, there was no significant variation in EC between January and March, but it differed significantly with the result of May. So the spatio-temporal variation of EC depends on the Ca and Mg salts concentration. These results were similar to those reported by Adil (2010) and Bhrdwaj *et al.* (2010). There was a significant spatial variation in EC between the two locations during the period of the study.

The results of TDS at both sites were followed consistently with the results of EC (Figures4 and 3). At site one, the value of TDS was 975 mg/l in May and 1364 mg/l in January. Whereas at site two, the minimum value was 605 mg/l in May and the maximum value was 621 mg/l in January and March (Table 1).

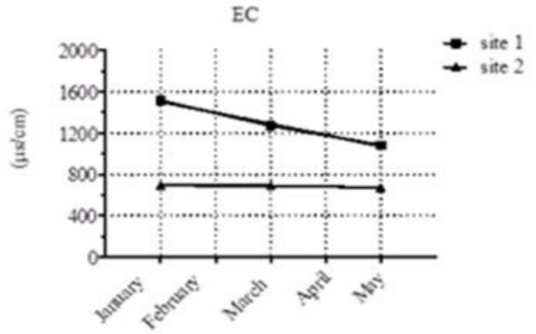


Figure 3. Electrical conductivity of water samples at the two sites during the period of the study

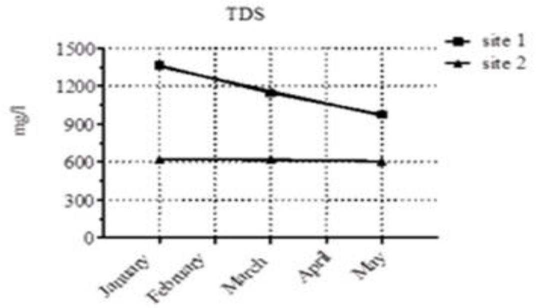


Figure 4. Total dissolved salts of water samples at the two sites during the period of the study

The temporal variation in site one was 389 mg/l and 16 mg/L in site two. In site one there was a significant temporal variation in the TDS during the periods of study, while in site two no significant variation in the results of TDS was found between January and March but it differed significantly from that of May. There was a significant spatial variation in TDS between the two sites during the period of the study. The spatio-temporal variation of TDS was due to the climate, geological formation and the time of sampling (Allan and Castillo, 2007; Aloisie *et al*., 2008).

The pH values of water at both sites were alkaline, and this is a characteristic of the freshwater in Kurdistan Region because calcium carbonate is the main component of the geology formation of the area, which is mainly composed of calcium carbonate (Ezat, 2002; Toma, 2006; Adil, 2010). At site one, the values of pH ranged from 8.08 to 8.33,in which the minimum value was recorded in January, whereas the maximum value was recorded in May (Table 1 and Figure 5). The temporal variation in this site was 0.25.ThepH value in January varied significantly with the pH in March and May. While in site two the minimum value was 7.21 in March and the maximum value was 7.50 in January. So the temporal variation at this site was 0.259. Statistically, the results in site two were similar to those of site one. There was a significant spatial variation in pH value between the two sites throughout the period of the study. These results are in agreement with the results of many researchers at Kurdistan region (Toma, 2006; Hamasalh, 2008), who found that the pH of water ecosystem in the region was alkaline and this is due to geological formation of the area. According to the water criteria and standards for pH, the pH of the two sites are coincident with the Environmental Protection Agency (EPA) in which the pH of most unpolluted surface water is generally between 6.5-8.5, and the pH of natural unpolluted ground water is generally between 6.0-8.5. For aquatic life the pH should be 6.5-9 and should not vary more than 0.5 units beyond the normal seasonal maximum and minimum (Eugene, 2008).

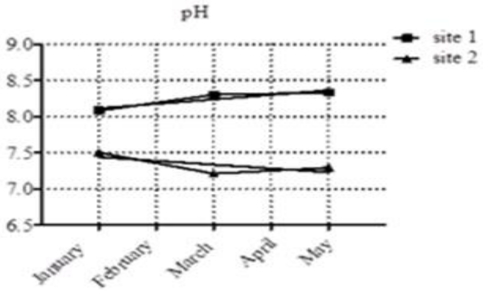


Figure 5. pH of water samples at the two sites during the period of the study

The results of DO at site one were 6.5 mg/l in May and 7.0 mg/l in January (Table 1 and Figure 6). The maximum value was in January because the water temperature was low and the water was turbulent in winter with a high flow, whereas the minimum value was in May because the water temperature was high with a low flow water and high salts content (Oprean *et al.*, 2008; Barbaro, 2008). No significant temporal variation was found in the DO in site one during the study period. As forsite two,there was a very low temporal variation in the value of DO which was 3.8 mg/l in May and the maximum value was 4.5 mg/l in January. In site two, a very low temporal variation was noticed in the value of DO (0.7 mg/L),which was due to the fact that the underground water lacks DO (Allan and Castillo, 2007).There was a significant temporal variation in the values of DO during the times of sampling at site two. The spatial variation showed a significant difference during the period of the study, and this was due to the water temperature, partial pressure of Oxygen in the water and the salts content of water (Oprean *et al*., 2008; Barbaro, 2008).

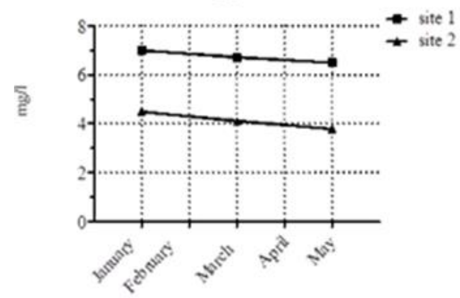


Figure 6. Dissolved oxygen water samples at the two sites during the period of the study

Water alkalinity in site one was 226mgCaCO$_3$l^{-1} in May and 260 mg CaCO$_3$l^{-1} in January, whereas at site two it was 156mgCaCO$_3$l^{-1} in May and 197mgCaCO$_3$l^{-1} in January (Table 1 and Figure 7). A significant temporal variation was found between May and that of January and March in both sites, while a significant spatial variation was noticed throughout the period of the study, which can be attributed to the surface water and groundwater draining from carbonate mineral formation, becoming more alkaline in January because of the increase in the carbonate minerals dissolution (Eugene, 2008;Hasan *et al*.,2009).According to the quality and standards for alkalinity, naturally occurring levels of alkalinity reaching at least 400mgCaCO$_3$l^{-1} are not considered a health hazard (Eugene, 2008). Also, according to the results, it is clear that there was a small temporal variation in the values of alkalinity and this reflects the values of pH and the total hardness at both sites.

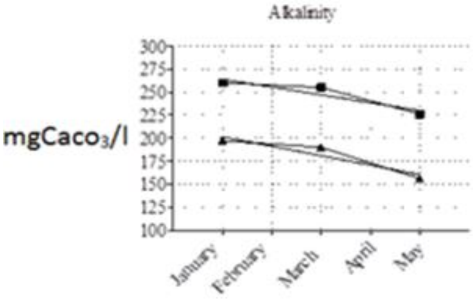


Figure 7. Alkalinity of water samples at the two sites during the period of the study

Measuring hardness is useful as an indicator for the Total Dissolved Solids (TDS). Ca, Mg, CO_3^{-2} and HCO_3^- form the largest part of the total hardness. The values of total hardness at site one varied from 460 to 552 mg CaCO$_3$l^{-1} (Figure 8) and at site two from 271 to 291mg CaCO$_3$l^{-1}. At site one; the highest value was in January, whereas the lowest value was in May. The temporal variation at site one was 92mg CaCO$_3$l^{-1}, whereas at site two it was 20 mg CaCO$_3$l^{-1}. This temporal variation is due to the discharge and the speed of water flow which cause more dissolution of Ca and Mg salts (Allan and Castillo, 2007). The highest and the lowest values of hardness at site two were recorded in March and May, respectively.

It is obvious from the results of the total hardness at site two that temporal variation was very narrow (Figure 8), because the underground water was characterized by a limited variation in its physical and chemical characteristics (Aloisie *et al*., 2008).

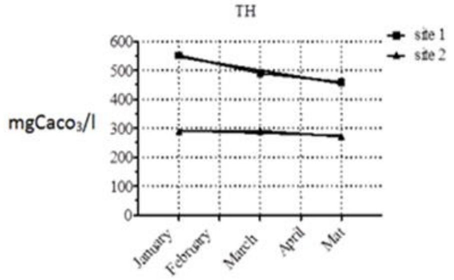


Figure 8. Total hardness of the water samples at the two sites during the period of the study

The result of the total hardness at both sites was coincident with the results of EC and TDS (Figures 3 and 4). According to the classification of Spellman (2008), the water at site one was very hard and at site two it was hard. There was a significant temporal variation in the values of the total hardness in site one during the period of the study, whereas in the site two no significant difference was found in the values of the total hardness between January and March but they differed significantly from those of May. Also, statistical analysis showed that there was a significant spatial variation between the two sites during the period of the study.

Mg hardness was slightly more than the Ca hardness at site one. The values of Ca hardness varied from 195 in May to 260 mg CaCO$_3$l^{-1}in January and the values of Mg hardness varied from 252 in March to 292mg CaCO$_3$l^{-1} in January (Table 1, Figures 9 and 10). At site two, Ca hardness was more than the Mg hardness, and this hardness was due to the dominance of Ca ions, and its value varied from 175mg CaCO$_3$l^{-1} in May to 212 CaCO$_3$l^{-1} in March, whereas the Mg hardness varied from 79mg CaCO$_3$l^{-1} in March to 96mg CaCO$_3$l^{-1} in May.

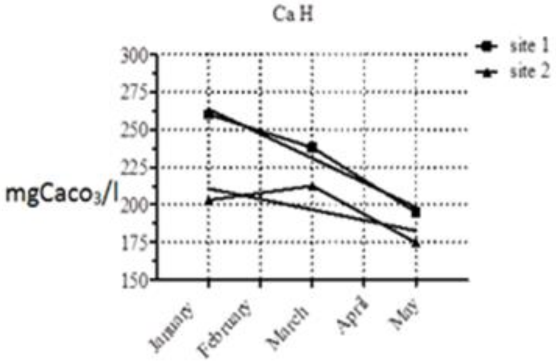


Figure 9. Calcium hardness of the water samples at the two sites during the period of the study

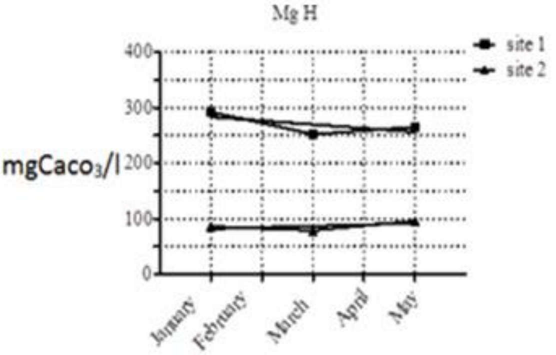


Figure 10. Magnesium hardness of the water samples at the two sites during the period of the study

The results of Ca ions and Mg ions reflect the results of Ca and Mg hardness. The Ca ions values at site one varied from 78 mg/l in May to 104.2 mg/l in January. While in site two, it varied from 70 in May to 85 mg/l in March (Figure 11). Mg ions at site one varied from 61 mg/l in March to 70.9 mg/lin January, while at site two they varied from 19 mg/l in March to 23 mg/l in May (Figure 12).A significant spatio-temporal variation was found in the values of Ca hardness at both sites throughout the period of the study. In site one; there was a significant temporal variation in the values of Mg ions during the period of the study, while Mg ion values in May were significantly differed from those in January and March at site two.

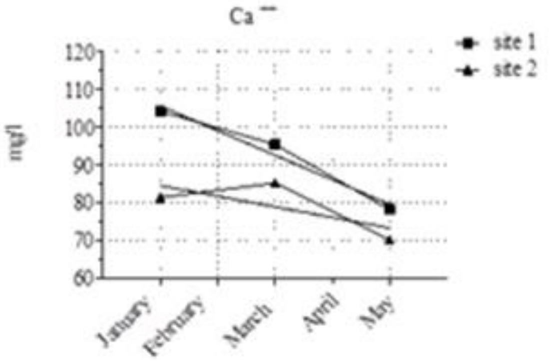


Figure 11. Calcium ions of the water samples at the two sites during the period of the study

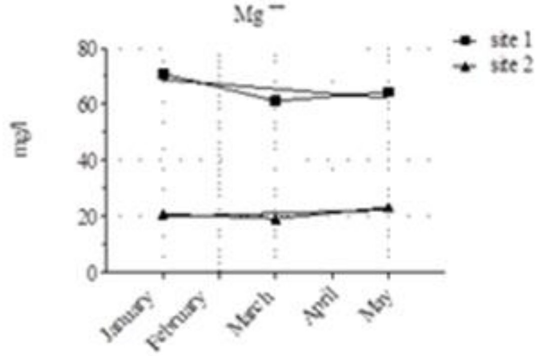


Figure 12. Magnesium ions of the water samples at the two sites during the period of the study

Nitrate values varied from 104 µg /l in March to 191 µg/l in January at site one, whereas at site two they were212 µg/l in March and 335 µg/l in January. A high temporal variation was found at site two (123 µg/l), while less temporal variation was found at site one which was 87 µg/l. So the fluctuation of nitrate concentration during the period of the study was very clear at both sites in which the low concentration was found in March and the high concentration was found in January. A high concentration of nitrate in springs is very common and it was found in many parts of the world (Odum, 1971). In Kurdistan Region, Eza t(2002) found that the nitrate concentrations in some springs at Duhok region ranged from 10 µg/l to 80 ugl^{-1}. The high concentration of nitrate in January is expected because of the high deranging of water through the different parts of its catchments area, and the lower value of nitrate was recorded in March because of the low deranging of water from the aquifer; this is explained by David (1996).Statistically, there was a significant spatio-temporal variation in nitrate concentrations during the period of the study (Table 1 and Figure 13).

Concerning the results of phosphate(Table 1andFigure14), the values of phosphate at site one were 30 µg/l in March and 97.8 µg/l in January, while at site two they were 28µg/l in March and 84.4 µg/l in January. So the results at both sites are coincident and the low values at both sites were recorded in March and the high values in January. The concentration of both nitrate and phosphate increased in January which is due to the high deranging of water at both sites (Veenie, 1999; Ezat, 2002). Also, the results showed that the low spatial variation was in March and in May, and it was quite high in January; generally there was a marginal variation in the values of phosphate between the two regions. A significant spatio-temporal variation in soluble reactive phosphate was found in both sites.

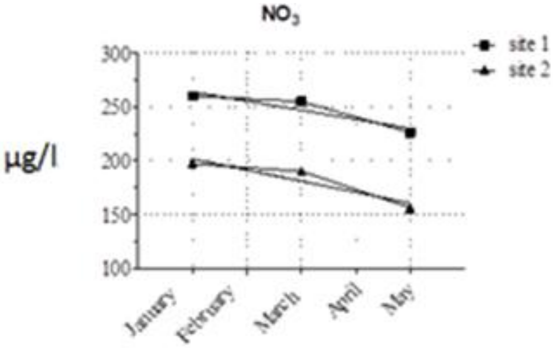


Figure 13. Nitrate ions of the water samples at the two sites during the period of the study

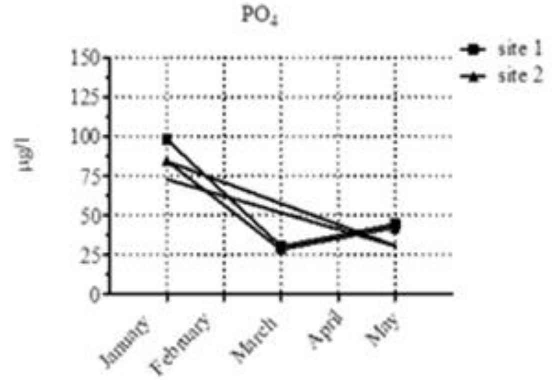


Figure 14. Orthphosphate ion concentrations of the water samples at the two sites during the period of the study

Algal Communities

A total of twenty species belonging to ten genera of four algal divisions (Chlorophyta, Cyanophyta, Baccllariophyta and Rhodophyta) were recorded (Table 2).Identified algal flora in the present study included epipelic, epilithic and epiphytic algae. Although Aloisie (2008) found that the Benthos algae in freshwater habitats were mainly dominated by cyanobacteria, green algae, diatoms and red algae, but the present study did not show this fact. In site one, the algal composition was dominated by diatoms and at site two only one species of red algae and one species of blue green algae were found during the period of the study. From the results of algal flora (Table 2), there was no temporal variation at both sites, whereas a spatial variation was very conspicuous between the two sites. At site one the algal flora included *Cladophora glomearat* and *Zygogonnium ericetarum* belonging to Chlorophyta, *Oscillatoria simplicissima*, *Oscillatoria formosa*, *Oscillatoria limnetica* and *Oscillatoria srubescens* belonging to Cyanophyta, *Cymbellacy biformis var. nonpuctata* (Plate 3-C), *Cymbella turgid* (Plate 3-B), *Cymbella ventricosa* (Plate 3-A), *Cymbella affinis*,*Diatoma hiemale*, *Diatoma anceps var. linearis*

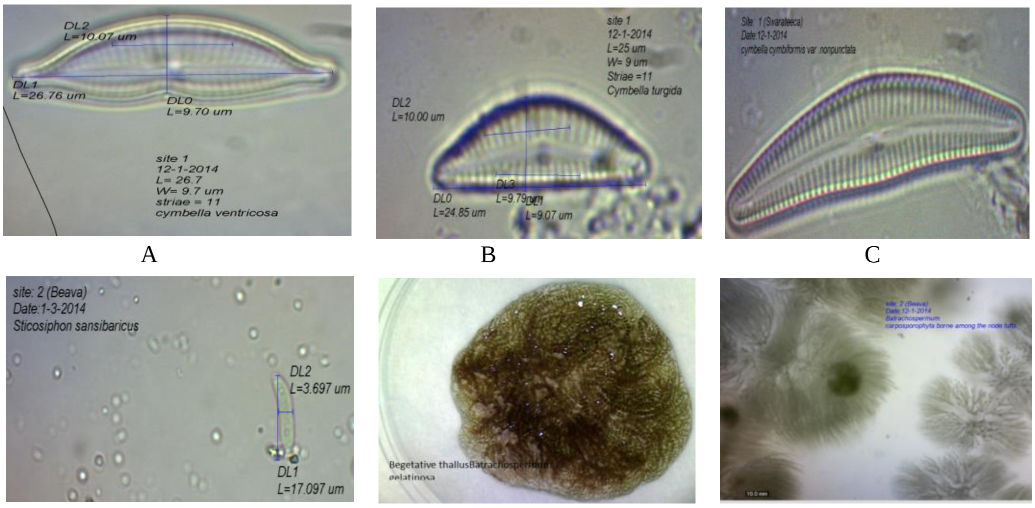
(Plate 3-E), *Diatoma vulgare*,- *Rhopalodia gibba*, *Rhopalodia gibberula* and *Navicula sp.* belonging to the Bacillariophyta.

At site two, during the period of sampling only one taxon was identified which was *Batrachospermum gelatinosia* (Plate 3- F) belonging to the Rhodophyta, and in March one taxon was identified which was *Sticosiphon sansibaricus* belonging to the Cyanophyta (Plate 3-D). Mature carposporphyte of *Batrachospermum gelatinosa* was noticed in March (Plate 3 G and H ;Plate 4).

A surprising result was noticed at site two in which yellowish green color covered the stones at the bed of the spring's pool. This yellow green color was due to the dense growth of bacteria which were Gram negative bacilli, motile, oxidase positive and non-sugar fermentative. The primary diagnosis was *Pseudomonas* species. All isolates were tested against 45 different biochemical tests using Phaenix ID system (BD Diagnostic Systems, Sparks, MD), and the diagnosis was *Pseudomonas fluorescens* with a confidence value 96.Fluorescent *Pseudomonas* strains constitute a diverse group of bacteria that can be distinguished from other Pseudomonads by their ability to produce water-soluble yellow-green pigment (Dabboussi *et al.*; 1999).Dense growth of *Pseudomonas fluorescens* as a biofilm covering the stones prevented the growth of other types of algae because of the lytic- activity of this bacteria (Jeong *et al.*,2007)(Table 2).

Table 2. Showing the identified microbentheic and macrobenthic algae at two sites during the period of study.

Locations	Time of sampling		
	January,2014	March	May
Site 1	1-*Cladophora glomearat:* 2-*Cymbella cybiformis var. nonpuctata* 3-*Cymbella turgida* 4-*Cymbella ventricosa* 5-*Cymbella affinis* 6-*Diatoma hiemalis* 7-*Diatoma anceps var. linearis* *8-Diatoma vulgare.* Note :*Diatoma vulgare* densely epiphytic on *Cladophora glomearat* Note: epilithic sample of diatoms was dominantly *Diatoma vulgaris* and *Cymbella* spp. and *Synedra ulna*were rarely found.	1-*Cymbella cymbiformis* 2-*Cymbella affinis* 3-*Cymbella microcephala* 4- *Synder ulna* 5-*Diatoma anceps* Note: the following algae were found far from the source of water: 1-*Oscillatoria simplicissima* 2-*Oscillatoria formosa* 3-*Oscillatoria limnetica* 4-*Oscillatoria rubescens*	1-*Zygogonnium ericetarum* 2- *Cymbella affinis* 3-*Cymbella cymbiformis* 4-*Cymbella minuta* 5- *Synedra ulna* 6- *Rhopalodia gibba* 7-*Rhopalodia gibberula* 8-*Naviculaspp.*
Site 2	1-*Batrachospermum gelatinosia*	1-*Batrachospermum gelatinosia:* mature cystocarpus observed in March. Also in this month the alga present in dense form on the stones at the bed of springs pool. 2-*Sticosiphon sansibaricus*	1-*Batrachospermum gelatinosia*



A B C

D E F

Plate 3. Cymbella ventricosa (A), *Cymbella turgida* (B), *Cymbella cymbiliformis* var. *nonpunctata* (C), *Sticosiphon sansibaricus* (D),Vegetative thallus of *Batracospermum gelatinosa* (E), F mature carposporophyte of *Batracospermum gelatinosa*.

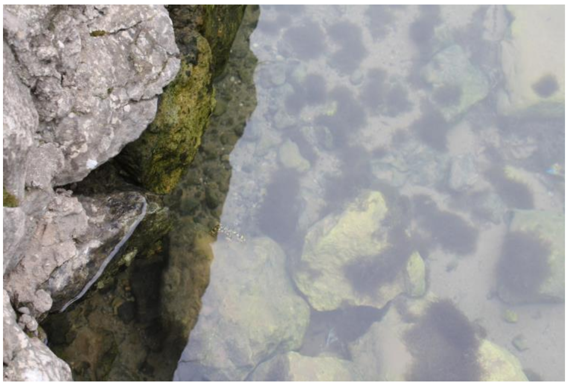


Plate 4. View of Site two showing *Batrachosperum gelatinosia* covered the stones in the bottom of the pool.

4. Conclusion

In site one, there were significant differences between all physico-chemical parameters except for DO and alkalinity, while a less significant difference was observed at site two. Presence of *Neurergus crocatus* salamander in the aquatic ecosystem indicates low algal species richness and low nitrate and phosphate concentrations in the water. The algae, listed in Table 2,can grow in oligotrophic aquatic ecosystem. Only *Batrachospermum gelatinosa* can resist the lytic activity of *Pseudomonas fluorescens.* The spatial variation in the structure of algal community between both sites was clearly evident. No temporal variation was noticed in algal community at each site during the period of the study.

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In vitro Biochemical Assessments of Methanol Stem Bark Extracts of *Ficus sycomorus* Plant

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Abstract

Natural antioxidants are important in disease prevention and promotion of health. We determined antioxidants' activity using DPPH radical scavenging activity, Hydrogen peroxide radical scavenging activity and Ferric reducing antioxidants power. The extracts significantly ($p< 0.05$) exhibited strong antioxidants activities at concentrations (20, 40, 60, 80 and 100 mg/ml) compared to L-Ascorbic. Preliminary phytochemical screening showed the presence of tannins, saponnins, flavonoids, terpenoids, phenols and steroids while glycoside and proteins were absent. The extracts significantly ($p< 0.05$) exhibited a receptor binding affinity when estimated using hemolytic inhibition assay and hemagglutination inhibition assay at 20, 40, 60, 80 and 100 mg/ml on human erythrocytes. Antibacterial activity (% Inhibition) was tested against five pathogenic organisms (*E. coli*, *S. aureus*, *S. typhi*, *B. cereus and P. aeruginosa*). The extracts significantly ($p< 0.05$) inhibited *E. coli* (30.82 ± 8.73) at 2 mg/ml, *S. aureus* (30.23 ± 6.56, 40.31 ± 2.88 and 43.38 ± 0.94) and *S. typhi* (30.49 ± 0.81, 36.99 ± 0.50 and 47.69 ± 1.02) at 2 mg/ml, 4 mg/ml and 6 mg/ml, respectively, and *P. aeruginosa* (41.82 ± 1.12, 49.02 ± 0.34, 56.03 ± 0.50, 69.90 ± 0.27 and 73.26 ± 0.43) at 2 mg/ml, 4 mg/ml, 6 mg/ml, 8 mg/ml and 10 mg/ml compared to standard drug. The standard drug significantly ($p< 0.05$) inhibited *B. cereus* (28.82 ± 0.80, 40.40 ± 0.40, 44.92 ± 1.20, 56.20 ± 2.52 and 62.72 ± 0.79) at 2 mg/ml, 4 mg/ml, 6 mg/ml, 8 mg/ml and 10 mg/ml compared to the stem bark extracts.

Keywords: **:** Antioxidant activity, Antibacterial activity, Antihemolytic assay, *Ficus sycomorus*

1. Introduction

Antioxidants are substances that protect living cells from the damage caused by unstable molecules known as free radicals. Antioxidants are known to interact and stabilize free radicals thereby preventing damage. The free radical damage may lead to the development of cancer (Prior *et al.*, 2005). Antioxidant molecules are capable of slowing or preventing the oxidation of other molecules. Oxidation refers to the chemical reaction that transfers electrons from one substance to another. Oxidation reactions produce free radicals which start chain reactions that damage the cells. Antioxidants terminate these chain reactions by removing free radical intermediates and inhibiting other oxidation reactions. Examples of some antioxidants are Beta-carotene, lycopene, vitamins A, C and E (Lopez *et al.*, 2007).

One major role of antioxidants is protecting the living cells from potentially damaging oxidative stress resulting from imbalance between the formation of Reactive Oxygen Species (ROS) and the body antioxidant defense. Naturally occurring antioxidants are used in foods because of their potential in health promotion, disease prevention, high safety and consumer acceptability. Antioxidants are used in food industry to prevent deterioration, nutritional losses and off-flavoring in various foods, especially those containing polyunsaturated fatty acids (Gorinstein *et al.*, 2003).

Ficus sycomorus Linn belongs to the family of *Moraceae*, comprising about 40 *genera* and over 1,400 species of trees, shrubs, vine and herbs, often with milky latex juices. The plant grows up to 20 m with widely spreading branches and crown. *F. sycomorus* fruits, stem bark and root are widely used in Nigeria, Niger, Mali, South Africa, Guinea, Kenya, Tanzania, Somalia, Ethiopia and Ivory Coast for the treatment of various diseases such as cough, diarrhea, skin infections, stomach disorders, liver disease, epilepsy, tuberculosis, lactation disorders, helminthiasis, infertility, sterility and diabetes mellitus (Igbokwe *et al.*, 2010; Adoum *et al.*, 2012). The plant has also been reported to be a potent antimicrobial agent against ciprofloxacin resistant *Salmonella typhi* (Adeshina *et al.*, 2010). *F. sycomorus* extracts have been reported by Auda (2012) for the treatment of various skin diseases while the decoction is used for treating

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gastrointestinal tract problems. It is further used as seasoning; the stem bark is dried and added to cake as a condiment, eaten raw or cooked as soup. Nkafamiya *et al.* (2010) reported that the proximate analysis of *F. sycomorus* plant showed high contents of proteins and crude fibers while ash, lipid and carbohydrate contents were within the range expected for dry leafy vegetables. In view of the above background, the present study seeks to investigate the antioxidants activities, hemolytic inhibition assay, hemagglutination inhibition assay and antibacterial activities of methanol stem bark extract of *F. sycomorus*

2. Materials and Methods

2.1. Collection of Plant Material

Fresh stem bark of *F. sycomorous* plant was collected from around Sangere, Girei Local Government Area, Adamawa State. Sangere is located on latitude 9^0 $11^{'}$ $15^{''}$ N and longitude 12^0 $20^{'}$ $29^{''}$E, on the North bank of River Benue. The plant was taxonomically identified and authenticated in the Plant Science Department of Modibbo Adama University of Technology, Yola. The stem bark was air dried in the laboratory for 7 day and thereafter made into powder using electric blender. The coarse material was sieved using 0.3 mm Endicott test sieve.

2.2. Preparation of the Plant Extract

Air dried and powdered plant material 300 g was extracted with methanol by cold extraction process for 24 h with intermittent stirring. The solvent extract was filtered using a sterilized Whatman filter paper No.1 to obtain a particle free extract. The solvent extract was concentrated by evaporation of the solvent at < 50^0C using rotary evaporator and vacuum oven to obtain dry powder. The extract was stored until use.

2.3. Qualitative Phytochemical Screening

Qualitative phytochemical screening of the freshly prepared crude extract was tested for the presence of carbohydrates, alkaloids, flavonoids, steroids, phenols, tannins, saponnins, glycosides and proteins as described by Nweze *et al.* (2004) and Senthilkumar and Reetha, (2009).

2.4. Determination of DPPH (2, 2-diphenyl-2-picryl hydrazyl) Radical Scavenging Activity

The DPPH radical scavenging capacity of the plant extracts was determined according to the method described by Sasidharan *et al.* (2007). The free radical scavenging activity of the extract was measured by the decrease in absorbance of methanol solution of DPPH. The DPPH radical scavenging method was used for the determination of the antioxidant capacity of the extracts. A different concentration of the plant extracts (20, 40, 60, 80 and 100 mg /ml, in methanol) was added at an equal volume (10 ml) to methanol solution of DPPH (400 µg/ml). A different concentration of L-Ascorbic acid (20, 40, 60, 80 and 100 mg /ml) was used as the standard antioxidant. The antioxidant activity of the stem bark extract was compared with L-Ascorbic acid. IC_{50} values (where 50 % of the radicals were scavenged by the test sample) were interpolated from the reference inhibition curve. After 30 min incubation at room temperature, the absorbance values were measured at 517 nm on a spectrophotometer (VIS 721, PEC MEDICAL USA) and converted into the percentage antioxidant activity using the following equation:

DPPH antiradical scavenging capacity (%)

$$= \frac{\text{Absorbance of sample} - \text{Absorbance of blank}}{\text{Absorbance of blank}} \times 100$$

2.5. Hydrogen Peroxide Scavenging Assay

The hydrogen peroxide scavenging activity was determined using the method of Repon *et al.* (2013). A solution of hydrogen peroxide (40 mmol/l) was prepared in phosphate buffer (50 mmol/l, pH 7.4). The concentration of hydrogen peroxide was determined by absorption at 230 nm using a spectrophotometer. Extract (20-100 mg/ml) in phosphate buffer was added to hydrogen peroxide and absorbance at 230 nm was determined after 10 minutes against a blank solution containing a phosphate buffer without hydrogen peroxide. L-Ascorbic acid was used for comparison. The percentage of hydrogen peroxide scavenging was calculated using the following:

(%) of H_2O_2 scavenging activity

$$= \frac{\text{Absorbance of control} - \text{Absorbance of test}}{\text{Absorbance of control}} \times 100$$

while Ascorbic acid was used as a positive control.

2.6. Ferric Reducing Antioxidant Power (FRAP assay)

In ferric reducing antioxidant power assay, various concentrations (20, 40, 60, 80 and 100 mg/ml) of the methanol extracts were mixed with 1 ml of 0.2 M sodium phosphate buffer (pH 6.6) and 1 ml of 1% potassium ferricyanide in separate test tubes. The reaction mixtures were incubated in a temperature controlled water bath at 50^0C for 20 minutes followed by the addition of 1 ml of 10% trichloroacetic acid. The mixtures were then centrifuged for 10 minutes at room temperature. The supernatant obtained (1 ml) was added with 1 ml of deionized water and 200 µl of 0.1% $FeCl_3$. The control was prepared in the same manner as the samples except that 1% potassium ferricyanide was replaced by distilled water. The absorbance of the reaction mixture was measured at 700 nm. L-ascorbic acid was used as a standard. The reducing power was expressed as an increase in $A_{700\ nm}$ after blank subtracted (Bancrejee *et al.*, 2008). Percentage inhibitory activity was calculated using the following:

(%) Inhibitory activity

$$= \frac{\text{Absorbance of control} - \text{Absorbance of test}}{\text{Absorbance of control}} \times 100$$

while Ascorbic acid was used as a standard.

2.7. Anti-hemolytic Assay

Inhibition of H_2O_2 induced red blood cell hemolysis of methanol extract was determined as described by Tavazzi *et al.* (2001). The erythrocytes, from human blood, was separated by centrifugation and washed with saline or isotonic sodium phosphate buffer (pH 7.4) until the supernatant becomes colorless. The erythrocytes were then diluted with saline or phosphate buffer to give a 4%

suspension. Varying amounts of the extract (20, 40, 60, 80 and 100 mg /ml), with saline or buffer, was added to 2 ml of the suspension of erythrocytes and the volume was made up to 3.5 ml with saline or buffer. This mixture was pre-incubated for 120 min and then 0.5 ml H_2O_2 solutions of appropriate concentration in saline or buffer was added. The concentration of H_2O_2 in the reaction mixture was adjusted so as to bring 90% hemolysis of blood cells after 120 m in incubation. Incubation was being concluded after these time intervals by centrifugation during 5 min at ×1000 g and the extent of hemolysis was determined by the measurement of the absorbance at 540 nm corresponding to hemoglobin liberation. The anti-hemolytic activity was expressed as the inhibition percentage and was calculated using the following formula:

Anti-hemolytic activity (%)

$$= \frac{\text{Control 540 nm} - \text{Sample 540 nm}}{\text{Control 540 nm}} \times 100$$

where, $\text{Sample}_{540\text{ nm}}$ was the absorbance of the sample and $\text{Control}_{540\text{ nm}}$ was the absorbance of the control.

2.8. Hemagglutination Inhibition Assay

The hemagglutination activity of the methanol stem bark extracts of *F. sycomorus* plant was tested against human erythrocyte blood groups A^+, B^+, AB^+ and O^+ as described by Saha *et al*. (2009). Stock solution of the test samples was prepared at concentration of 20, 40, 60, 80 and 100 mg/ml and each solution was serially diluted. Fresh blood from healthy volunteers was collected, centrifuged and the erythrocytes were separated. 4% erythrocyte suspension was prepared in phosphate buffer (pH 7.4) for all blood groups. One ml of the extract dilution was taken with 1 ml of 4% erythrocyte and incubated at 4 °C. After incubation, the results were noted. Smooth formation in the bottom indicated a negative activity, while a rough granular deposition at bottom showed a positive activity. The intensity of hemagglutination was determined from the extent of deposition.

2.9. Antibacterial Activity

The antibacterial activity of the methanol stem bark extracts of *F. sycomorus* plant was determined as described by Akinyemi *et al*. (2005) using the modified broth dilution technique method. Five test tubes were dispensed with 2 ml of sterile Nutrient broth followed by addition of 0.1ml of standardized inoculums of test organisms (*E. coli*, *S. aureus*, *S. typhi*, *B. cereus and P. aeruginosa*) to each test tube. Various concentrations (2, 4, 6, 8 and 10 mg/ml) of the stem bark extracts were added, and the test tubes were incubated aerobically at 37^0C for 18-24 hr. Two control tubes were then maintained for each batch. These include the antibiotic control (antibiotic, growth medium and organism) and organism control (growth medium only and test organism). The antibacterial activity of the *F. sycomorus* extracts, antibiotic control and organism control were read using a colorimeter at 490 nm. Percentage growth inhibition was computed using the following given formula:

Percentage growth Inhibition (**C**)

$$= 100 - \frac{\text{Absorbance of sample}}{\text{Absorbance of control}} \times 100$$

2.10. Statistical Analysis

Results are reported as mean ± SD. The difference between two means was analyzed using one-way analysis of variance (ANOVA). Significance was taken at $p < 0.05$. SPSS version 20 (USA) was used for the analysis.

3. Results

3.1. Phytochemical Screening

The preliminary phytochemical screening of *F. sycomorus* stem bark extract (Table 1) showed the presence of tannins, saponnins, flavonoids, glycosides, proteins, phenols terpenoids and steroids while glycosides and proteins were absent.

Table 1. Phytochemical composition of methanolic stem bark extracts of *F. sycomorus* plant

Phytochemicals	*F. sycomorus* stem bark
Tannins	+
Saponnins	+
Terpenoids	+
Flavonoids	+
Glycosides	-
Proteins	-
Phenols	+
Steroids	+

Key: Present: +, Absent: -

3.2. Determination of DPPH Radical Scavenging Activity

Figure 1 shows the result of the DPPH radical scavenging activity of *F. sycomorus* stem bark extract. The result shows that the stem bark extracts significantly ($p < 0.05$) exhibited a radical scavenging activity compared to L-Ascorbic acid. The half maximum inhibitory concentration (IC_{50}) of the stem bark extract and L-Ascorbic acid were interpolated graphically and found to be 24.02 mg/ml and 20.00 mg/ml, respectively.

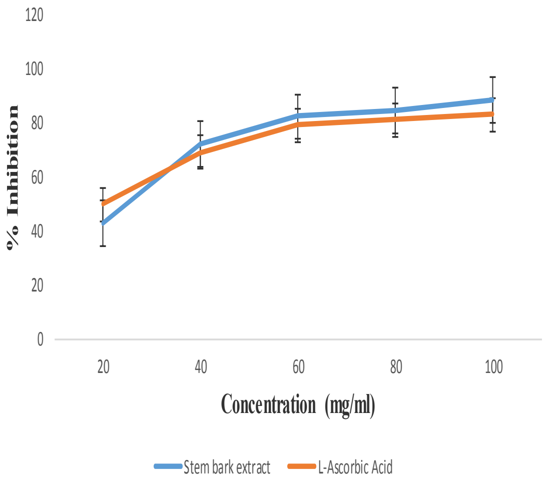

Figure 1: DPPH radical scavenging activity of methanol stem bark extract of *F. sycomorus* plant. Each values is expressed as mean ± SD, n = 3, ($p < 0.05$).

3.3. Hydrogen Peroxide Radical Scavenging Activity

The hydrogen peroxide radical scavenging activity of the stem bark extract was measured in comparison with L-ascorbic acid (Figure 2). Results showed that the stem bark extract significantly ($p < 0.05$) exhibited an antioxidant radical scavenging activity compared to the L-ascorbic acid.

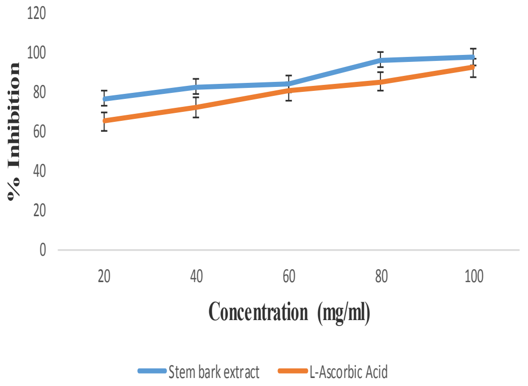

Figure 2: Hydrogen peroxide scavenging activity of methanol stem bark extract of *F. sycomorus* plant. Each value is expressed as mean ± SD, n = 3, ($p < 0.05$)

3.4. Ferric Reducing Antioxidant Power

The antioxidant activity determined using Ferric reducing antioxidants power showed that the stem bark extract significantly ($p < 0.05$) exhibited a stronger antioxidant activity compared to L-ascorbic acid (Figure 3) in a dose-dependent manner. The IC_{50} of the stem bark extract and L-Ascorbic acid were interpolated graphically as 28.0 mg/ml and 33.05 mg/ml, respectively

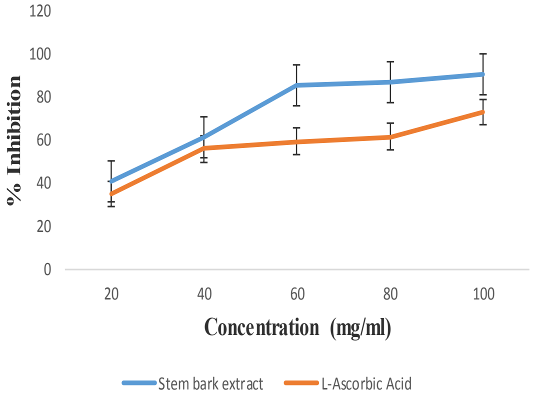

Figure 3: Ferric reducing antioxidant power of methanol stem bark extract of *F. sycomorus* plant and L-ascorbic acid. Each values is expressed as mean ± SD, n = 3, ($p < 0.05$)

3.5. Hemolytic Inhibition Activity

Hemolysis induced by hydrogen peroxide in red blood cell (Figure 4) showed that the plant extract inhibited hemolysis at various concentrations of the extract used. The results showed decreases in hemolysis as extracts concentration increases in a dose-dependent manner.

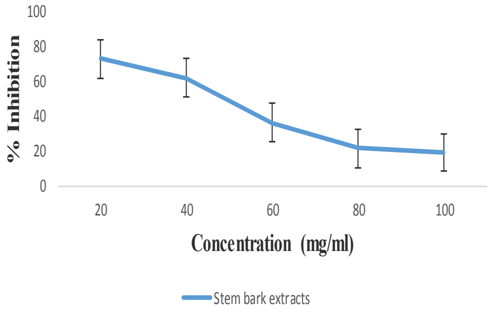

Figure 4: Hemolytic inhibition activity of *F. sycomorus* stem bark extracts. Values are expressed as mean ± SD, n = 3, ($p < 0.05$)

3.6. Hemagglutination Inhibition Assay

The hemagglutination inhibition assay (Table 2) of different human blood groups was determined using various concentrations of *F. sycomorus* stem bark extract (20 - 100 mg/ml). The result showed low, moderate and strong activity of the extracts at 60 mg/ml, 80 mg/ml and 100 mg/ml for blood groups A$^+$, B$^+$, AB$^+$ and O$^+$ while no activity was observed at lower doses except for blood group B$^+$ at 40 mg/ml.

Table 2. Hemagglutination inhibition assay of stem bark extract of *F. sycomorus* plant

Blood group	20 mg/ml	40 mg/ml	60 mg/ml	80 mg/ml	100 mg/ml	Buffer only
A+	_	_	_	+	+	_
B+	_	+	+	++	++	_
AB+	_	_	+	+	++	_
O+	_	_	+	++	+++	_

-: No activity; +: low activity; ++: moderate; +++: strong activity

3.7. Antibacterial Assay

Antibacterial activities of *F. sycomorus* stem bark extract were tested against five selected pathogenic organisms (Table 3). The result showed the inhibition percentage of the extract compared to the antibiotic drug Azithromycin at various concentrations of the extract. *E. coli* activity was significantly inhibited using 2 mg/ml extract (30.82 ± 8.73 %) compared to Azithromycin (14.69 ± 1.48 %). *S. aureus* and *S. typhi* activity were significantly inhibited by the extract at 2 mg/ml (30.23 ± 6.56 % and 30.49 ± 0.81 %), 4 mg/ml (40.31 ± 2.88 % and 36.99 ± 0.50 %) and 6 mg/ml (43.38 ± 0.94 % and 47.69 ± 1.02 %), respectively, while the antibiotic Azithromycin significantly inhibited the tested organisms at higher concentrations:8 mg/ml (55.53 ± 1.23 % and 71.48 ± 1.11 %) and 10 mg/ml (63.37 ± 0.82 % and 75.00 ± 1.22). Azithromycin showed high inhibition against *B. cereus* compared to the stem bark extract while the pathogenic organism *P. aeruginosa* activity was significantly inhibited (41.82 ± 1.12 %, 49.02 ± 0.34 %, 56.03 ± 0.50 %, 69.90 ± 0.27 % and 73.26 ± 0.43 %) by the stem bark extract at the various concentrations used compared to the standard drug Azithromycin.

Table 3. Antibacterial activities (% Inhibition) of *F. sycomorus* stem bark extract

	Concentration	*E. coli*	*S. aureus*	*S. typhi*	*B. cereus*	*P. aeruginosa*
Stem bark extract	2 mg/ml	30.82 ± 8.73*	30.23 ± 6.56*	30.49 ± 0.81*	22.64 ± 1.63	41.82 ± 1.12*
	4 mg/ml	27.53 ± 2.18	40.31 ± 2.88*	36.99 ± 0.50*	32.79 ± 1.72	49.02 ± 0.34*
	6 mg/ml	30.85 ± 3.16	43.38 ± 0.94*	47.69 ± 1.02 *	44.57 ± 0.29	56.03 ± 0.50*
	8 mg/ml	43.63 ± 4.47	54.62 ± 2.76	53.47 ± 0.60	53.71 ± 0.29	69.90 ± 0.27*
	10 mg/ml	68.16 ± 6.98	62.25 ± 1.10	73.61 ± 1.06	57.33 ± 1.27	73.26 ± 0.43*
Azithromycin	2 mg/ml	14.69 ± 1.48	12.50 ± 0.41	18.40 ± 0.81	28.82 ± 0.80	14.12 ± 1.60
	4 mg/ml	37.50 ± 4.02	13.20 ± 3.03	21.77 ± 1.52	40.40 ± 0.40	18.68 ± 1.22
	6 mg/ml	42.05 ± 0.80	32.40 ± 2.24	23.57 ± 2.44	44.92 ± 1.20	41.95 ± 0.81
	8 mg/ml	59.66 ± 0.81	55.53 ± 1.23	71.48 ± 1.11	56.20 ± 2.52	42.24 ± 1.21
	10 mg/ml	64.21 ± 0.80	63.37 ± 0.82	75.00 ± 1.22	62.72 ± 0.79	55.46 ± 2.04

Values are Mean ± SD (N = 5); * Significant increased ($p < 0.05$) compared to Azithromycin

4. Discussion

Medicinal plants have a wide variety of phenolic compounds, such as flavonoids that act potentially as antioxidants, scavenging free radicals, reactive oxygen species and inhibit lipid peroxidation (Kumawat *et al.*, 2012). Antioxidants activities of stem bark extracts of *F. sycomorus* using DPPH radical scavenging activity, hydrogen peroxide scavenging activity and ferric reducing antioxidants power showed that the extracts significantly ($p < 0.05$) exhibited strong antioxidants activity compared to the standard (L-Ascorbic Acid) at the concentrations used. The results support the use of the plant therapeutically as well as economically as antioxidant additives or nutritional supplements and explored for novel antioxidants (van Wyk, 2008). The antiradical activity of the extracts could be related to the high content of tannins and flavonoids. The effects of the extracts could be due to the biological systems that are linked to their ability to transfer electrons to free radicals, chelate metals, activate antioxidant enzymes, reducing radicals of alpha-tocopherol or to inhibit oxidases (Bruneton, 2009). The results support studies on antioxidant activity of *Ficus pyriformis* extract and MeOH fraction possessing good scavenging activity compared to reference standards (ascorbic acid and quercetin) (Zedan *et al.*, 2015). The MeOH fraction showed a maximum activity in comparison with the other fractions. The antioxidants activity exhibited by the extract could be due to the presence of poly-phenolic compound such as flavonoids. The presence of ortho-dihydroxyl of the B-ring (3', 4'-di OH) of flavonoid molecule confers high stability to the flavonoid phenoxy radical, C2-C3 double bond in conjugation with 4-oxo group of the ring C participates in radical stabilization via electron delocalization over all three ring system. The presence of both 3- and 5-hydroxyl moiety of the rings C and A play an important role in radical scavenging activity of the flavonoids (Lv *et al.*, 2013).

Studies on phytochemical components confirmed the presence of tannins, saponnins, flavonoids, terpenoids, phenols and steroids while glycoside and proteins were absent. The presence of these constituents may be attributed to the antioxidants activity of the extracts. Medicinal plants contain different phytochemicals with biological activities having valuable therapeutic index. Therapeutic effects of this medicinal plant are attributed to the presence of their phytochemicals which are non-nutrient plant compounds. The major phytochemicals revealed are known to possess a wide range of activities, which may help in protection against chronic diseases. Saponnins, flavonoids, tannins and alkaloids are known to posses hypoglycemic and anti-inflammatory activities, saponnins also possess hypocholesterolemic and antidiabetic properties (Augusti *et al.*, 2008) while terpenoids decreases blood sugar levels in animal studies, steroids, triterpenoids and saponnins showed analgesic properties and central nervous system activities (Argal *et al.*, 2006).

The antihemolytic inhibition assay of the extracts showed inhibitory effect on hydrogen peroxide induced hemolysis. The result showed a decrease in hemolysis as the concentration of the extract increases. The inhibitory effect may be attributed to their phenolic compounds which can donate electrons to H_2O_2, thus neutralizing it to water molecule (Alinezhad *et al.*, 2012). The antihemolytic inhibition of the extract was exhibited in a dose-dependent manner. Hemagglutination inhibition assay of the extracts exhibited a moderate (++) and a strong activity (+++) at a higher concentration (80 and 100 mg/ml) against the blood groups. The extracts may contain lectins and could be a useful source of important phytolectins. The hemagglutination activity is usually attributed to a group of proteins called lectins which are valuable agents for the separation and characterization of glycoconjugates and glycopeptides (Khan *et al.*, 2005).

The antibacterial activity (% inhibition) of the extract was tested against some organisms. The results revealed that the extracts significantly ($p < 0.05$) inhibited *S. aureus* and *S. typhi* at 2, 4, and 6 mg/ml, respectively. *P. aeruginosa* activity was significantly ($p < 0.05$) inhibited by the extract at 2, 4, 6, 8 and 10 mg/ml while the extract significantly ($p < 0.05$) inhibited *E. coli* at 2 mg/ml. The antibacterial activity exhibited could be due to the presence of phytochemicals (flavonoids, saponins, terpendois, phenol and tannins) and the occurrence of phenolic compounds in the extract (Ramde-Tiendrebeogo *et al.*, 2012).

5. Conclusion

Studies on the phytochemical, antioxidant, antihemolytic, hemagglutination inhibition and antibacterial activities of *F. sycomorus* stem bark extract showed that the extract is a beneficial sources of phytochemicals. The results showed that the extract exhibited a strong antioxidant activity compared to L-ascorbic acid. Hemolytic inhibition assay and hemagglutination inhibition showed that the extract has a strong receptor binding affinity on erythrocytes. The plant extract exhibited a strong antibacterial activity against the human pathogenic organisms investigated. The study, therefore, supports the therapeutic uses of the plant in traditional medicine and suggest the need to isolate, identify and characterize the active principles responsible for its activity.

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Non-Starch Polysaccharide Degrading Gut Bacteria in Indian Major Carps and Exotic Carps

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Abstract

Cellulose and xylan are the most common Non-Starch Polysaccharides (NSPs); they are available in plants and they exhibit anti-nutritional effects. The present study is intended to detect cellulose and xylan degrading autochthonous gut bacteria in Indian major carps (*Labeo rohita*, *Catla catla*, and *Cirrhinus mrigala*) and exotic carps (*Hypophthalmichthys molitrix*, *Ctenopharyngodon idella*, and *Cyprinus carpio*); it is also meant to identify the most promising strains by molecular methods. The promising strains were also tested for likely antagonism against few pathogenic *Aeromonas* strains. Altogether, 432 microbial strains were isolated on media containing either cellulose or xylan. Seventy strains were primarily selected through qualitative enzyme assay. Finally, the quantitative assay led to the selection of 5 promising NSP-degrading strains (LFR1X, CMF1C, HMF6X, CtIF1C, and CMH8X). Amongst these, LRF1X was the best cellulase- and xylanase-producer. Analyses of 16S rRNA partial gene sequence revealed that strains LRF1X and CMF1C were closely related to *Bacillus pumilus* (Accession numbers; KF640221, KF640223, respectively), whereas HMF6X, CtIF1C, and CMH8X were similar to *B. tequilensis* (KF640219), *B. megaterium* (KF640220) and *B. altitudinis* (KF640222), respectively. The culture of the selected microorganisms with autochthonous bacteria and yeasts indicated their co-existence within the fish gut. An appraisal of antagonism against four pathogenic *Aeromonas* species by the cross-streaking method revealed that the selected NSP-degrading strains (except CMH8X) were antagonistic to at least 2 pathogens. In vivo bio-safety assessment through intra-peritoneal injection of the isolates showed no induction of pathological lesions or mortality in healthy laboratory acclimatized rohu, *L. rohita.*

Keywords: **:** Cellulose, Xylan, Carps, Bacteria, Aeromonas.

1. Introduction

Non-Starch Polysaccharides (NSPs) are complex polysaccharides that are polymers of hexoses and pentoses (e.g., galactose, glucose, arabinose, xylose, mannose, etc.) excluding starch or α-glucans (Van Barneveld, 1999). The NSPs comprise up to 90% of the plant's cell wall (Selvendran and DuPont, 1980), wherein cellulose, hemicelluloses, and pectins are the most abundant (Sinha *et al*., 2011). Cellulose is the basic structural component of plant cell walls and constitutes about 33% of all vegetable materials. Xylan is the most common hemicellulose and represents the major non-cellulosic cell wall polysaccharide in plants. In contrast, pectic polysaccharides (pectins) are only present in modest amounts in plants (Sinha *et al*., 2011). Being an integral part of plant ingredients, NSPs are represented in aquaculture through natural food and supplementary feeds in the form of phytoplankton, algae, aquatic macrophytes, detritus, husks (rice bran or wheat bran) and different oil cakes. The predominant endogenous polysaccharide digesting enzymes, in fish specifically, hydrolyze the α-glycosic linkages of starch to yield glucose. Presence of β-(1→4) glycosidic linkages is the reason why cellulose is indigestible by monogastric animals due to the lack of the enzyme cellulase in their gastrointestinal (GI) tract. Similarly, the other enzymes for NSP digestion, such as β-glucanases and β-xylanases, are also either scarce or not present in fish (Kuz'mina, 1996). Consequently, the dietary NSPs remain mostly indigestible and cannot be used as a nutritional source. In addition, being partially soluble in water, NSPs increase the viscosity of the digesta, leading to changes in the physiology and the ecosystem of the gut, thus, exerting anti-nutritive effects (Sinha *et al*., 2011). Therefore, it appears that unless hydrolyzed or degraded by exogenous enzymes, NSPs would decrease the nutritive value of the plant feedstuffs.

The contribution of endosymbionts in the digestive process requires understanding the relative importance of both, endogenous (produced by self) and exogenous (produced by endosymbionts) enzymes (Clements, 1997).

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During the last few decades, there has been an improved understanding of the enzymes produced by the resident endosymbiotic microbial community in fish (Ringø *et al*., 2010). Several studies have reported the occurrence of cellulase-producing microorganisms within the GI tract of diverse fish species, including carps.[1]

In contrast, reports on xylanase production by fish gut microorganisms are scarce (German and Bittong, 2009; Banerjee and Ghosh, 2014). In the previous studies, association of cellulase (Saha and Ray, 1998; Bairagi *et al*., 2002; Ghosh *et al*., 2002, 2010; Saha *et al*., 2006; Mondal *et al*., 2008), phytase (Roy *et al*., 2009; Khan *et al*., 2011; Khan and Ghosh, 2012) and tannase (Mandal and Ghosh, 2013) producing microorganisms were demonstrated as an ecological adaptation of the carps towards herbivory. Likewise, the existence of the gut-associated symbiotic microbial community capable of producing NSP-degrading enzymes (in addition to cellulase) could not be excluded in carps.

Carps are the freshwater fish of the family Cyprinidae. Various species of carps have been included in aquaculture as food fish across Europe and Asia for centuries. Three Indian major carp species (*Labeo rohita*, *Catla catla*, and *Cirrhinus mrigala*) along with three Chinese carps (*Hypophthalmichthys molitrix*, *Ctenopharyngodon idella*, and *Cyprinus carpio*) constitute the composite carp culture in India. All these carp species are mostly herbivorous or omnivorous in feeding aptitude (Jhingran, 1997). Intensive carp polyculture demands improvements in feed efficiency, while the replacement of animal protein sources (e.g., fish meal) with the non-conventional plant ingredients is already a concern to trim down the production costs. Enumeration of exo-enzyme producing gut microorganisms capable of degrading complex polysaccharides and their utilization as either feed supplement or *in vitro* processing of plant feedstuffs has been recommended in some of the recent investigations (Khan and Ghosh, 2013; Das and Ghosh, 2015).

Since, cellulose and xylans are the major NSPs in plant feedstuffs, the present study is undertaken to appraise the occurrence of cellulase and xylanase-producing autochthonous microorganisms within the GI tracts of six freshwater carps. The most promising enzyme-producing strains were characterized through 16S rRNA partial gene sequence analysis to corroborate their identity. Bio-safety in fish was evaluated *in vivo* by injecting healthy laboratory acclimatized rohu, *L. rohita*, with fresh cultures of the isolates. In addition, the promising enzyme-producers were evaluated for antagonism against few well-known fish pathogens belonging to the genus *Aeromonas*. Motile aeromonads, being the major bacterial pathogens among tropical freshwater fish, are reported to be the main causative factor behind mass mortalities associated with increased stocking in composite carp culture (Karunasagar *et al*. 1986). *Aeromonas* outbreaks in aquaculture are common in tropical countries and, therefore, the use of pathogen inhibitory microorganisms has been suggested as probiotic bio-control agents to substitute the use of antimicrobial drugs (Balcázar *et al*., 2006; Dutta and Ghosh, 2015). Finally, the selected bacterial isolates were cultured with the previously isolated autochthonous gut bacteria and yeasts to substantiate their co-existence as gut microbiota in fish in view of their likely application in aquaculture.

2. Materials and Methods

2.1. Experimental fishes

Six freshwater carp species consisting of three Indian major carps (rohu, *Labeo rohita*; catla, *Catla catla*; mrigal, *Cirrhinus mrigala*) and three exotic carps (silver carp, *Hypophthalmichthys molitrix*; (grass carp, *Ctenopharyngodon idella*; common carp, *Cyprinus carpio*) were selected for the present study. Three specimens of each species were collected by gill net from three different composite fish culture farms at and around Burdwan (23°14'N, 87°39'E), West Bengal, India during June to September, 2012, and kept in 350L Fiber-Reinforced Plastic (FRP) aquaria. Specimens were brought to the laboratory within oxygen-packed bags. Descriptions of the fishes examined along with their feeding habits are depicted in Table1.

Table 1. Food habits, average live weight, average fish length, average gut weight and gut length of the fishes examined.

Fish Species	Food habits*	Average fish live weight (g)	Average fish length (cm)	Average gut weight (g)	Gut length (L_G) (cm)
Rohu, *L.rohita*	Omnivorous, mostly plant matter	260±13.44	30.5±2.61	11.32±0.62	271.7±8.51
Catla, *C.catla*	Zooplanktophagous	370±10.97	29.4±2.34	12.18±0.59	224.5±7.76
Mrigal, *C. mrigala*	Detrivorous	330±12.33	30.7±2.70	8.29±0.57	431.3±10.27
Silver carp, *H. molitrix*	Phytoplanktophagous	440±14.42	26.6±3.84	8.38±0.68	218.3±8.68
Grass carp,*C. idella*	Herbivorous, mostly macrophytes	450±10.88	28.9±2.21	16.7±0.55	63.2±8.39
Common carp, *C. carpio*	Detrivorous	375±13.44	27.4±2.37	7.81±0.58	47.3±9.81

Data are means ± S.D. of three determinations. * adapted from Jhingran (1997)

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[1] For more information, see Ray *et al.* (2012).

2.2. Processing of Specimens

Prior to sacrifice, experimental fishes were starved for 48 h to clear their gastrointestinal (GI) tracts and to remove traces of any undigested food or fecal matter therein (Mondal *et al.*, 2010). Specimens were anesthetized using 0.03% tricainemethanesulfonate (MS-222). Ventral surfaces were sterilized using 70% ethanol and fishes were dissected aseptically to remove the GI tracts (Ghosh *et al.*, 2010). GI tracts were divided into proximal (PI) and distal (DI) regions, cut into pieces, and flushed carefully three times with Sterile Saline Solution (SSS) using an injection syringe in order to remove non-adherent (allochthonous) microbiota (Ghosh *et al.*, 2010; Khan and Ghosh, 2012). Gut segments from 3 specimens of each species, collected from the same pond, were pooled together region-wise for each replicate, therefore providing 3 replicates for each fish species. Pooled samples were utilized to avoid erroneous conclusions due to individual variation in gut microorganisms as described elsewhere (Ringø *et al.*, 1995; Spanggaard *et al.*, 2000; Ringø *et al.*, 2006).

2.3. Microbial Culture

Pooled segments for each replicate were homogenized separately with pre-chilled SSS (1:10; weight: volume), serially diluted (1:10) up to 10^{-7}, and used as inoculums for isolation of gut microorganisms (Beveridge *et al.*, 1991). Diluted samples (100 µL) were spread aseptically onto sterilized tryptone soya agar (TSA; HiMedia Laboratories, Mumbai, India) plates to obtain the culturable heterotrophic aerobic/facultative anaerobic autochthonous microbial population. In order to isolate cellulase and xylanase-producing microorganisms, diluted samples were plated onto carboxy-methyl-cellulose (CMC, gL^{-1}: carboxy-methyl-cellulose 5, Yeast extract 5, peptone 5, NaCl 5, agar 20) and xylan (XY, gL^{-1}: peptone 5, yeast extract 2, $MgSO_4.7H_2O$ 0.5, NaCl 0.5, $CaCl_2$ 0.15, Birchwood xylan 20, agar 20) supplemented selective media plates as described in Dutta and Ghosh (2015). The culture plates were incubated at 30°C for 24 h. Colony counts were determined utilizing the dilution plate count technique. The average values of the replicates were expressed as log viable count g^{-1} GI tract (LVC). The well-separated colonies were randomly collected with inoculation loop and streaked individually onto respective media plates repeatedly to acquire pure cultures. Pure cultures were maintained on slants in a refrigerator (4°C) for further study.

2.4. Screening of Potent Cellulase and Xylanase-Producing Isolates by Qualitative and Quantitative Enzyme Assay

Gut isolates were primarily evaluated for a qualitative determination of extracellular cellulase and xylanase-producing capacities following growth (30°C, 48 h) on the selective media plates containing respective substrates. The cellulase-producing capacity was determined on CMC plates flooded with Congo red prepared with 0.7% agarose (Teather and Wood, 1982). Congo red selectively binds with unhydrolyzed carbohydrate polymers. The appearance of a clear zone (halo), due to the presence of hydrolyzed CMC, indicated a cellulase production in the medium. Positive xylanolytic isolates were distinguished after flooding the XY plates with Congo red solution [0.5% Congo red (w/v) and 5% ethanol (v/v)] 5 min., followed by repeated decolorizaion with 1 M NaCl (Ninawe *et al.*, 2006). The appearance of halo, owing to the hydrolyzed XY surrounding the bacterial colony, indicated xylanase production in the medium. Isolates producing a halo of ≥10 mm in excess of colony growth in each case were selected for quantitative enzyme activity.

Proficient NSP-degrading strains were identified through the quantitative assay of the extracellular cellulase and xylanase production. Respective selective broth media were used to obtain the enzymes. The culture flasks were incubated (37°C, 72 h) with vigorous shaking (150–170 rpm), centrifuged at 10,000*g* (4°C, 10 min), and the cell-free supernatant containing the enzymes was collected (Bairagi *et al.*, 2002). The quantitative assay for cellulase production was performed following the method described by Denison and Koehn (1977) using 1% CMC in sodium citrate buffer (0.1 M, pH 5.0) as substrate. Xylanase activity was assayed using 1% birch-wood xylan as the substrate as described by Bailey *et al.* (1992) and using D-xylose as the standard. Production of reducing sugar (glucose) from the substrate due to cellulolytic or xylanolytic activity was measured at 540 nm by the dinitrosalicylic acid method (Miller, 1959) using glucose or D-Xylose as the standard. Unit activity (U) of cellulase was defined as the µg of glucose liberated mL^{-1} of enzyme extract min^{-1}. Xylanase activity (U) was defined as the mg of D-xylose liberated mL^{-1} of enzyme extract min^{-1}.

2.5. Identification of Isolates by 16S rRNAGene Sequence Analysis

The most promising NSP-degrading strains (bacteria) were identified through 16S rRNA partial gene sequence analysis after isolation and PCR (polymerase chain reaction) amplification following the methods described in Das *et al.* (2014). To prepare template DNA, pure colonies were suspended in sterilized saline, centrifuged (12,000*g*, 10 min), supernatants removed and the pellets suspended in InstaGene Matrix (Bio-Rad, USA). DNA isolation was carried out following the manufacturer recommendations. PCR amplification of the 16S rRNA gene was performed using universal primers, 27f (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492r (5′-GGTTACCTTGTTACGACTT-3′). PCR was executed using a PCR mix containing 200 µM of deoxynucleotides (dNTPs), 0.2 µM of each primer, 2.5 mM $MgCl_2$, 1X PCR buffer, 0.2 U of Taq DNA polymerase (Invitrogen) and 1 µL of template DNA. The cycle used for PCR was: initial denaturation at 95°C for 3 minutes, followed by 35 cycles of denaturation at 94°C for 45 sec, annealing at 55°C for 1 min, extension at 72°C for 1 min, and a final extension at 72°C for 3 minutes (Lane, 1991). *E. coli* genomic DNA was included as positive control. PCR products were purified using Montage PCR Clean up kit (Millipore, USA). The sequencing of the purified PCR products was performed using the Big Dye terminator cycle sequencing kit (Applied BioSystems, USA). Sequencing products were resolved on an automated DNA sequencing system (Applied BioSystems 3730XL, USA). Sequence data were edited using BioEdit Sequence

Alignment Editor (Version 7.2.0); then they were aligned and analyzed to find the closest homolog using Basic Local Alignment Search Tool (BLAST) in National Centre for Biotechnology Information (NCBI) GenBank and Ribosomal Database Project (RDP) databases. Sequences were deposited in the NCBI GenBank and accession numbers were obtained. A phylogenetic tree was constructed incorporating 16S rRNA partial gene sequences of the closest type strains using the MEGA 5.1Beta4 software following the Minimum Evolution Method.

2.6. Fish Pathogens and Culture Maintenance

Four fish pathogenic strains: *Aeromonas salmonicida* MTCC-1945 (AS), *Aeromonas sobria* MTCC-3613(AB), *Aeromonas hydrophila* MTCC-1739 (AH), and *Aeromonas veronii* (KT737240) (AV) were used to evaluate pathogen inhibitory activity of the promising cellulase and xylanase-producing strains. The pathogenic strains were maintained in the laboratory on T SA (HiMedia, Mumbai, India) slants at 4°C. Stock cultures in Tryptone Soya Broth (TSB) were stored at -20°C in 0.85% NaCl with 20% glycerol to provide stable inoculums throughout the study (Sugita *et al.*, 1998).

2.7. In Vitro Antagonistic Activity and Test of Compatibility

Pathogen inhibitory activity of the selected strains was studied against the four *Aeromonas* spp. utilizing the ‘cross-streaking’ method (Madigan *et al.*, 1997). The NSP-degrading bacterial isolates were cultured with previously isolated eight autochthonous fish gut bacteria and two yeasts: *Bacillus subtilis subtilis* (JX292128), *Bacillus atrophaeus* (HM246635), *Bacillus subtilis* (HM352551), *Bacillus pumilus* (KF454036), *Bacillus flexus* (KF454035), *Bacillus methylotrophicus* (KF559344), *Bacillus subtilis* subsp. *spizizenii* (KF559346), *Enterobacter hormaechei (KF559347)*, *Pichia kudriavzevii* (KF479403), and *Candida rugosa* (KF479404). Source and description of these autochthonous gut microorganisms were presented in previous studies (Das and Ghosh, 2013; Khan and Ghosh, 2012; Banerjee *et al*. 2015; Mukherjee and Ghosh 2014; Banerjee and Ghosh, 2014). Selected strains were inoculated as a line onto TSA media plates and incubated at 30°C for 24 h. Subsequently, pure cultures of the autochthonous fish gut microorganisms (8 bacteria, 2 yeasts) were inoculated as a perpendicular line to the NSP-degrading bacterial strains keeping a gap of about 1 mm. Following incubation (30°C, 24 h), the growth of microorganisms was examined and the disappearance of the gap indicated compatibility (co-existence) of the autochthonous yeast and bacteria strains with the NSP-degrading bacterial strains.

2.8. Safety of the Selected Isolate

The safety of the selected isolates (LRF1X, CMF1C, HMF6X, CtIF1C, and CMH8X) was evaluated through *in vivo* studies conducted in 350 L FRP tanks using 90 healthy *L. rohita* (average body weight: 15±1.7 g) divided into six groups (five experimental and one control), each with three replicates. The fish were acclimatized for 2 weeks in FRP aquaria under laboratory conditions. Group 1 fishes were kept as control, and each fish received Intra-Peritoneal (IP) injection of 1.0 mL SSS (Mesalhy *et al.*, 2008). The five selected NSP-degrading bacterial isolates were grown in Tryptone Soya Broth (TSB) at 30°C for 24 h, centrifuged (2800*g* for 15 min, 4°C), and cell pellets were suspended in SSS. Each fish in the experimental groups (groups 2, 3, 4, 5, and 6) was given IP injection of 1.0 mL saline containing 10^9 cells ml^{-1} of each test bacterium. All groups were kept under observation for 21 days for likely development of any disease symptom.

2.9. Statistical Analysis

Statistical analysis of quantitative enzyme activity data was performed using the analysis of variance (ANOVA) followed by Tukey’s test according to Zar (1999) using Statistical Package for the Social Sciences (SPSS) Version 10 (International Business Machines Corporation, Armonk, New York) (Kinnear and Gray, 2000).

3. Results

Enumeration of gut microbial communities in the GI tracts revealed that considerable amounts of autochthonous aerobic or facultative anaerobic culturable heterotrophic, as well as cellulose and xylan-degrading microorganisms, were present in both the PI and DI regions of all the fish species studied (Table 2). The highest counts of cellulase-producing bacteria were noted in the DI region of mrigal, C. mrigala (LVC=6.14 g^{-1} intestinal tissue), followed by the DI region of catla, C. catla (LVC=5.97 g^{-1} intestinal tissue). Similarly, the highest counts of xylanase-producing bacteria were noted in the DI region of silver carp, H. molitrix (LVC=5.34 g^{-1} intestinal tissue), followed by the DI region of rohu, L. rohita (LVC=5.04 g^{-1} intestinal tissue).

Table 2. Log viable counts (LVC) of cellulase and xylanase producing autochthonous (adherent) bacteria isolated from theproximal (PI) and distal (DI) parts of intestine of the fish species examined.

Fish species and intestinal site of isolation		LVC g^{-1} intestinal tissue		
		Heterotrophic count on TSA plate	Bacterial count on cellulase plate	Bacterial count on xylanase plate
L. rohita	PI	6.98	5.12	4.94
	DI	7.11	5.45	5.04
C. catla	PI	6.32	5.45	4.19
	DI	6.65	5.97	4.54
C. mrigala	PI	6.95	5.07	4.26
	DI	7.54	6.14	4.82
H. molitrix	PI	7.25	4.95	4.93
	DI	7.85	5.26	5.34
C. idella	PI	5.54	4.26	4.14
	DI	5.96	4.69	4.28
C. carpio	PI	6.48	4.65	3.81
	DI	6.89	4.86	4.56

Altogether, 432 microbial strains were isolated on the media containing either cellulose or xylan. Seventy strains were primarily selected through qualitative enzyme assay, from which 30 microbial strains were further studied by quantitative assay to select the most promising isolates.

Data pertaining to quantitative assay of cellulase and xylanase activities for the 30 promising strains (12 from PI and 18 from DI), 5 strains from each of the 6 fish species, are presented in Table 3. This led to the selection of 5 promising NSP-degrading strains (LFR1X, CMF1C, HMF6X, CtIF1C, and CMH8X)

Table 3. Quantitative assay of the enzyme activity (unit activity, U) by the selected bacterial isolates. Numbers within the parenthesis denote total numbers of isolates from the respective fish species examined.

Source and number of isolates*	Strains$^{\#}$	Activity of NSP-degrading enzymes	
		Cellulase†	Xylanase^{+}
L. rohita (PI-38, DI-47)	LRH4X	55.48 ± 2.48^{f}	12.38 ± 1.21^{d}
	LRF1X	64.61 ± 2.39^{g}	24.25 ± 1.29^{g}
	LRH3C	44.55 ± 1.12^{e}	7.07 ± 0.26^{b}
	LRH5C	48.61 ± 1.04^{e}	9.26 ± 0.29^{c}
	LRH5X	49.14 ± 1.19^{e}	8.81 ± 0.31^{c}
C. catla (PI-25, DI-39)	CCF1C	17.34 ± 1.62^{ab}	11.38 ± 0.44^{d}
	CCF1X	20.41 ± 1.71^{b}	7.59 ± 0.65^{b}
	CCF2X	21.51 ± 1.64^{b}	7.67 ± 0.54^{b}
	CCF3X	23.58 ± 1.81^{b}	8.48 ± 0.54^{bc}
	CCH4C	19.36 ± 1.64^{ab}	7.73 ± 0.59^{b}
C. mrigala (PI-30, DI-35)	CMF1C	36.51 ± 1.26^{d}	15.41 ± 1.17^{f}
	CMF3C	13.35 ± 1.08^{a}	15.18 ± 1.06^{f}
	CMH2C	16.73 ± 0.75^{a}	10.58 ± 0.72^{cd}
	CMH3X	20.68 ± 1.01^{b}	11.43 ± 1.06^{f}
	CMH8X	27.62 ± 1.25^{c}	16.34 ± 1.21^{f}
H. molitrix (PI- 34, DI-50)	HMF1C	24.34 ± 1.11^{bc}	10.24 ± 0.27^{c}
	HMF1X	23.44 ± 1.25^{b}	8.13 ± 0.45^{bc}
	HMF6X	48.46 ± 2.15^{d}	23.28 ± 1.37^{g}
	HMH1C	23.53 ± 1.72^{b}	13.25 ± 1.27^{e}
	HMH5X	22.41 ± 1.29^{b}	8.23 ± 0.31^{bc}
C. idella (PI-26, DI-37)	CtIF1C	26.35 ± 1.21^{c}	13.41 ± 1.29^{e}
	CtIF2C	24.46 ± 1.20^{bc}	11.33 ± 1.01^{d}
	CtIH1X	14.41 ± 1.23^{a}	8.95 ± 0.31^{c}
	CtIH2X	21.48 ± 0.94^{b}	8.14 ± 0.29^{bc}
	CtIH3X	29.14 ± 1.18^{c}	4.65 ± 0.28^{a}
C. carpio (PI-26, DI-45)	CyCH3C	22.26 ± 1.72^{b}	7.43 ± 0.31^{b}
	CyCH4C	17.89 ± 1.25^{ab}	6.84 ± 0.29^{a}
	CyCH5C	17.39 ± 1.19^{ab}	7.24 ± 0.27^{b}
	CyCH6C	21.76 ± 1.14^{b}	8.35 ± 0.26^{bc}
	CyFH5X	16.65 ± 1.14^{a}	6.66 ± 0.27^{b}

Values with the same superscripts in the same vertical column are not significantly different ($P < 0.05$).

*PI: Proximal intestine; DI: Distal intestine

$^{\#}$The alphabets 'F' and 'H' before the numeric value indicate isolates from PI and DI, respectively

†µg of glucose liberated mL^{-1} of enzyme extract min^{-1}

$^{+}$ mg of D-xylose liberated mL^{-1} of enzyme extract min^{-1}

The strain LRF1X, isolated from the PI of *L. rohita*, exhibited the highest cellulolytic activity (64.61±2.39 U). A considerable cellulase activity was also noted with other strains isolated from *L. rohita*. On the other hand, the highest xylanase activity was recorded in LRF1X (24.25±1.29 U), followed by the strain HMF6X (23.28±1.37 U). Strains CMF1C (15.41±1.17 U), CMH8X (16.34±1.21 U), and CtIF1C (13.41±1.29 U) also exhibited a substantial xylanase activity. Considering the cumulative activities of the two NSP-degrading enzymes, isolates LRF1X, CMF1C, HMF6X, CtIF1C, and CMH8X were finally selected for identification and for possible future evaluation of use.

Based on nucleotide homology and phylogenetic analysis of the 16S rRNA partial gene sequences and using the nucleotide blast in the NCBI GenBank and RDP databases, the strains LRF1X and CMF1C were identified as *Bacillus pumilus* (Accession no. KF640221 and KF640223, respectively), which were closest to the type strain *Bacillus pumilus* (Accession no. AY876289). Another isolate, HMF6X, was identified as *Bacillus tequilensis* (Accession no. KF640219) as it showed a maximum similarity with the type strain *Bacillus tequilensis* (Accession no. HQ223107). Likewise, NCBI GenBank and RDP databases revealed that CtIF1C and CMH8X were similar to *Bacillus megaterium* (Accession no. KF640220) and *Bacillus altitudinis* (Accession no. KF640222), respectively. The isolate CtIF1C showed a similarity with *Bacillus megaterium* (Accession no. D16273), while the isolate CMH8X was closest to *Bacillus altitudinis* (Accession no.AJ831842). The identities of the promising NSP-degrading fish gut bacteria and their homology with the closest type strains are presented in Table 4. Phylogenetic relation of the five identified cellulase and xylanase-producing bacteria with closely related type strains, retrieved from the RDP database, are presented in the dendogram (Figure 1).

To verify the pathogen inhibitory activity, five selected NSP-degrading bacterial isolates were screened against four different strains of fish pathogenic *Aeromonas* spp. Results of the pathogen inhibitory activity, as revealed by the cross streaking method, are depicted in Table 5. Four strains (LRF1X, CMF1C, HMF6X, and CtIF1C) were antagonistic towards two of the tested *Aeromonas* spp. The strain CMH8X showed antagonism only towards *Aeromonas veronii*. Three strains (LRF1X, CMF1C, and HMF6X) were antagonistic towards *Aeromonas salmonicida*.

An *in vitro* co-culture test against eight autochthonous fish gut bacteria and two yeast isolates revealed that the selected bacterial strains did not affect the growth of the autochthonous gut microbiota. Therefore, the selected NSP-degrading bacterial isolates were considered compatible with commonly occurring autochthonous fish gut microbiota.

After 21 days of a small-scale *in vivo* experiment involving an intra-peritoneal injection of the selected bacterial isolates, no pathological signs, disease symptoms, or mortalities were observed in either the experimental sets or the control set.

Table 4. Identities of the promising NSP-degrading fish gut bacteria and their homology with the closest type strains in RDP.

Strain(s)	Closest type strains in RDP	S_ab score	Homology levels (Max. indent)	Bacterial species	NCBI GenBank accession number(s)
LRF1X	*Bacillus pumilus* AY876289	0.967	99%	*Bacillus pumilus*	KF640221
CMF1C	*Bacillus pumilus* AY876289	0.971	99%	*Bacillus pumilus*	KF640223
HMF6X	*Bacillus tequilensis* HQ223107	0.972	99%	*Bacillus tequilensis*	KF640219
CtIF1C	*Bacillus megaterium* D16273	0.915	98%	*Bacillus megaterium*	KF640220
CMH8X	*Bacillus altitudinis* AJ831842	0.962	99%	*Bacillus altitudinis*	KF640222

Table 5. Inhibition area (mm) produced by the selected gut bacteria (in excess of colony growth) incross streaking method against the tested fish pathogens.

Strains	AH	AS	AB	AV
LRF1X	-	2	2	-
CMF1C	1	1	-	-
CMH8X	-	-	-	2
HMF6X	2	3	-	-
CtIF1C	-	-	2	1

AH: *Aeromonas hydrophila*; AS:*Aeromonas salmonicida*; AB:*Aeromonas sobria*;

AV: *Aeromonas veronii*;

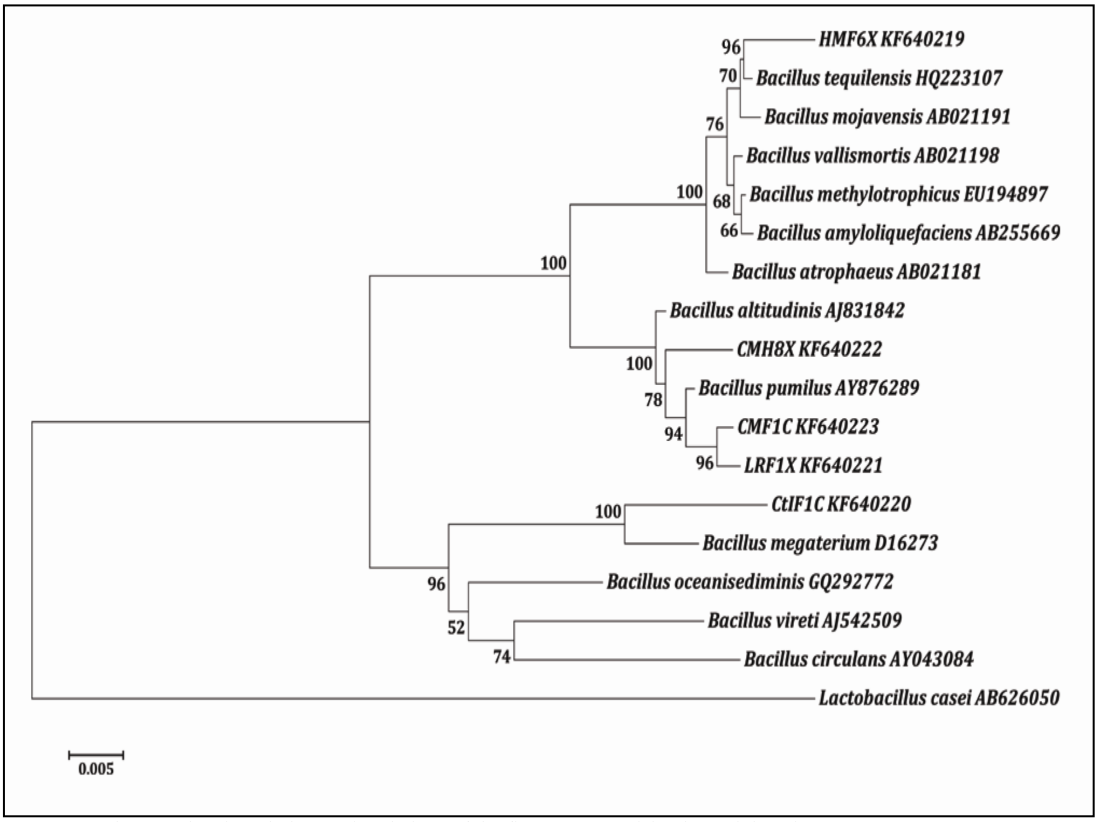

Figure 1. Dendogram showing phylogenetic relations of the five most promising bacterial strains, LFR1X, CMF1C, HMF6X, CtIF1C, and CMH8X, with other closely related type strains retrieved from NCBI GenBank. The GenBank accession numbers of the reference strains are shown in parentheses. Horizontal bars in the dendogram represent the branch length. Similarity and homology of the neighboring sequences have been indicated by bootstrap values. Distance matrix was calculated using Kimura 3-parameter model. The scale bar indicates 0.005 substitutions per nucleotide position. *Lactobacillus casei* AB626050.1 served as an out group control.

4. Discussion

Several plant-eating animals require the support of symbiotic microorganisms within their GI tracts to degrade complex polysaccharides or secondary metabolites to make the energy in these compounds available to the host (Karasov and Martinez del Rio, 2007). Likewise, the previous studies on fish advocated a possible degradation of starch (Ghosh *et al*., 2002, 2010), phytate (Roy *et al*., 2009; Khan *et al*., 2011; Khan and Ghosh, 2012), tannin (Mandal and Ghosh, 2013), and cellulose (Saha and Ray, 1998; Bairagi *et al*., 2002; Ghosh *et al*., 2002, 2010; Saha *et al*., 2006; Mondal *et al*., 2008) by the gut-associated microorganisms in diverse carp species. Although the existence of a cellulase activity in the digestive system of fish was in conflict with studies providing contradictory results, later on it became commonly accepted that fish lack the cellulase enzyme, as is the case with other monogastric and ruminant animals, with cellulose degradation being mediated through the action of cellulase produced by the fish gut microbiota (Ray *et al*., 2012). On the other hand, reports on xylanase production by fish gut endosymbionts are scanty. Many microorganisms, including bacteria (Balakrishnan *et al*., 2002; German and Bittong, 2009; Azeri *et al*., 2010), actinomycetes (Techapun *et al*., 2001; Tuncer *et al*., 2004), and filamentous fungi (Taneja *et al*., 2002; Angayarkanni *et al*., 2006; Sudan and Bajaj, 2007), have been reported to produce xylanase. Whether the fish gut sustains any autochthonous xylanase-producing microorganisms has not been authenticated yet, except for one report documenting the occurrence of xylanase-producing yeasts in some freshwater carps (Banerjee and Ghosh, 2014). Nonetheless, both cellulase and xylanase were assumed to be produced by microbes ingested by the fish with detritus rather than produced by a resident endosymbiotic community (German and Bittong, 2009). In the present study, heterotrophic as well as cellulase and xylanase-producing microbial symbionts were detected in the PI and DI regions of the GI tracts in 6 freshwater carp species studied. As the fish were starved for 48 h and their GI tracts were carefully cleansed with sterilized and cooled 0.9% saline prior to the isolation of microorganisms, it may be corroborated that the microorganisms isolated in the present study belonged to the autochthonous (adherent) microbiota as suggested elsewhere (Ghosh *et al*., 2010). The occurrence of a higher proportion of heterotrophic microorganisms in DI regions, compared to the PI regions, was in agreement with the previous reports (Mondal *et al*., 2008; Das *et al*., 2014). This suggests that the degradation of the feedstuffs within these parts of the GI tract occurs in assistance with the well settled enzyme-producing microbiota (Ghosh *et al*., 2010). The highest population counts of cellulase and xylanase-producing producing microbial symbionts were noted in the DI regions of mrigal, *C. mrigala* and silver carp, *H. molitrix*, respectively. Both species were either herbivores or feeding on detritus arising out from the plant feedstuffs (Jhingran, 1997). In addition, other carp species also harbored substantial amounts of NSP-degrading microorganisms within their GI tracts. Therefore, the presence of an appreciable quantity of culturable heterotrophic cellulase and xylanase-producing microbiota in both PI and DI regions of the GI tracts of the fish species studied might indicate their probable role in the degradation of NSPs in the plant feedstuffs.

Cereal by-products, oil cakes, and natural feeds like phytoplankton and aquatic weeds, contain considerable amounts of NSPs (cellulose and xylan) in their cell wall material. For instance, rice bran, generally used as an essential component in fish feed, contains approximately 20-25% NSP, consisting of approximately equal amounts of arabinoxylans and cellulose (Saunders, 1986). Anti-nutritional effects of NSPs in monogastric animals were generally associated with the viscous nature of the polysaccharides. High gut viscosity decreases the rate of diffusion of substrates and digestive enzymes and hinders their effective interaction at the mucosal surface (Edwards *et al*., 1988; Ikegami *et al*., 1990). The higher viscosity can reduce the rate of gastric emptying leading to satiety which then decreases the feed intake (Roberfroid, 1993). NSP degrading enzymes (e.g., cellulase and xylanase) cleave the large molecules of NSP into smaller polymers, thereby reducing the thickness of the gut content and increasing the nutritive value of the feed (Bedford *et al*., 1991; Choct and Annison, 1992).Therefore, the strategy to detoxify/degrade plant-derived anti-nutrients through the enzymes, produced by gut microbiota, might be regarded as an evolutionary adaptation as is the case for ruminant and non-ruminant herbivores (McBee, 1971).

As the major aim of the present investigation was to detect the efficient cellulase and xylanase-producing strains within the GI tracts of the freshwater carps (if any), the preliminary screening for extracellular cellulase and xylanase production has resulted in the elimination of 83.8% (362 out of 432) of the total isolates from current study. Furthermore, 5 efficient NSP-degrading strains were established through quantitative cellulase and xylanase assay and identified on the basis of 16S rRNA partial gene sequence analysis. The strains LRF1X and CMF1C were identified as strains of *Bacillus pumilus* (KF640221 and KF640223), while the strains HMF6X, CtIF1C, and CMH8X, were identified as *B. tequilensis* (KF640219), *B. megaterium* (KF640220), and *B. altitudinis* (KF640222), respectively. All promising NSP-degrading isolates belonged to *Bacillus* spp., which might support the hypothesis that gut bacteria in freshwater carps were dominated by diverse strains of *Bacillus* spp. (Ghosh *et al*., 2010; Ray *et al*., 2012). The occurrence of *B. pumilus* (Ghosh *et al*., 2002) and *B. megaterium* (Saha *et al*., 2006) within the gut of freshwater carps was reported previously. However, to the best of the authors' knowledge, *B. tequilensis* and *B. altitudinis* have not been documented from carp gut so far.

The abundance of cellulase-producing bacteria has been documented in the GI tracts of grass carp, *C. idella* (Bairagi *et al*., 2002; Saha *et al*., 2006; Li *et al*., 2009), common carp, *C. carpio* and silver carp, *H. molitrix* (Bairagi *et al*., 2002), rohu, *L. rohita* (Saha and Ray, 1998; Ghosh *et al*., 2002; Kar and Ghosh, 2008; Ray *et al*., 2010), catla, *C. catla* and mrigal, *C. mrigala* (Ray *et al*., 2010), and bata, *L. bata* (Mondal *et al*., 2008,2010).Furthermore, the xylanase-producing ability by gut inhabiting bacterial symbionts from freshwater carp species has not been documented to date. Results of the present study might suggest considerable opportunities for using cellulase as well as xylanase-producing bacterial symbionts from the gut of freshwater carps as aquaculture probiotics that might aid in the degradation of complex polysaccharides in feedstuffs within the gut microenvironment. In addition, *in vitro* degradation of NSPs in plant ingredients by autochthonus NSP-degrading bacteria could be an alternative approach of processing as the bacteria itself or their metabolites would not impair the normal function in fish because of the mutual relationship therein, as suggested previously for phytate-degrading gut bacteria (Khan and Ghosh, 2013).

Apart from nutritional benefits, the enzyme-producing gut bacteria in fish have been assumed to compete continuously with pathogens through the competitive exclusion or the production of antimicrobial compounds (Ray *et al*., 2012). Although several strains of Bacilli were demonstrated as probiotics for fish, antagonism of pathogens by the bacilli has been seldom indicated. *B. subtilis* SG4 (Ghosh *et al*., 2007) and *B. amyloliquefaciens* (Dutta and Ghosh, 2015) isolated from the gut of mrigal, *C. mrigala*, *B. methylotrophicus* isolated from channel catfish (Chao *et al*., 2012) and catla, *Catla catla* (Mukherjee and Ghosh, 2014) intestines, and *B. cereus* and *B. circulans* isolated from the gut of other fish species (Lalloo *et al*.,2010; Geraylou *et al*., 2014), were reported to exhibit pathogen inhibitory potential against different strains of *A. hydrophila*. In the present study, most of the promising NSP-degrading strains (4 out of 5) displayed antagonism against at least two of the four tested *Aeromonas* spp. To the best of the authors' knowledge, cellulase or xylanase-producing ability along with pathogen inhibitory potential of gut microflora from major carps depicted in the present study has not been reported previously. Furthermore, the test of compatibility revealed that the promising NSP-degrading strains did not affect the growth of previously isolated autochthonous fish gut bacteria and yeasts. Therefore, the present study might substantiate the likely co-existence of a diverse autochthonous gut microflora with the NSP-degrading strains as indicated in some preceding reports (Banerjee and Ghosh, 2014). The small scale in vivo experiment, involving the selected five strains, did not reveal any pathological lesions or mortality in the experimental fish model, *L. rohita*. Therefore, the *in vivo* application of the selected NSP-degrading strains, characterized in the present study, is considered safe, and could pave avenues for prospective future applications.

5. Conclusion

This preliminary study validates the occurrence of NSP-degrading bacterial symbionts within GI tracts of freshwater carps. However, autochthonous aerobic or facultative anaerobic gut bacteria were isolated in the present study by culture-dependent methods. Further studies involving anaerobic and culture-independent methods (such as Denaturing Gradient Gel Electrophoresis, Next Generation Sequencing, etc.) might be helpful in getting more information on the diversity of NSP-degrading microbiota within the fish GI tract. Furthermore, NSP-degrading and pathogen inhibitory bacteria, noticed in the present study, appear to provide the host with some ecological benefits by enabling them to conquer the adverse effects of NSPs and aeromonads. Whether the isolated gut bacteria can contribute to the host's nutrition and health has not been dealt with in the present study; a consideration of their function *in vivo* should be given high precedence in upcoming studies before advocating their utilization in commercial aquaculture.

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