Hashemite Kingdom of Jordan

Jordan Journal of Biological Sciences

An International Peer-Reviewed Scientific Journal

Financed by the Scientific Research Support Fund

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

ISSN 1995-6673

المجلة الأردنية للعلوم الحياتية
Jordan Journal of Biological Sciences (JJBS)
http://jjbs.hu.edu.jo

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

Submission Address

Professor Abu-Elteen, Khaled H
The Hashemite University
P.O. Box 330127, Zarqa, 13115, Jordan
Phone: +962-5-3903333 ext. 4357
E-Mail: jjbs@hu.edu.jo

المجلة الأردنية للعلوم الحياتية
Jordan Journal of Biological Sciences (JJBS)
http://jjbs.hu.edu.jo

International Advisory Board

Associate Editorial Board

Instructions to Authors

Scope

Study areas include cell biology, genomics, microbiology, immunology, molecular biology, biochemistry, embryology, immunogenetics, cell and tissue culture, molecular ecology, genetic engineering and biological engineering, bioremediation and biodegradation, bioinformatics, biotechnology regulations, gene therapy, organismal biology, microbial and environmental biotechnology, marine sciences. The JJBS welcomes the submission of manuscript that meets the general criteria of significance and academic excellence. All articles published in JJBS are peer-reviewed. Papers will be published approximately one to two months after acceptance.

Type of Papers

The journal publishes high-quality original scientific papers, short communications, correspondence and case studies. Review articles are usually by invitation only. However, Review articles of current interest and high standard will be considered.

Submission of Manuscript

Manuscript, or the essence of their content, must be previously unpublished and should not be under simultaneous consideration by another journal. The authors should also declare if any similar work has been submitted to or published by another journal. They should also declare that it has not been submitted/ published elsewhere in the same form, in English or in any other language, without the written consent of the Publisher. The authors should also declare that the paper is the original work of the author(s) and not copied (in whole or in part) from any other work. All papers will be automatically checked for duplicate publication and plagiarism. If detected, appropriate action will be taken in accordance with International Ethical Guideline. By virtue of the submitted manuscript, the corresponding author acknowledges that all the co-authors have seen and approved the final version of the manuscript. The corresponding author should provide all co-authors with information regarding the manuscript, and obtain their approval before submitting any revisions. Electronic submission of manuscripts is strongly recommended, provided that the text, tables and figures are included in a single Microsoft Word file. Submit manuscript as e-mail attachment to the Editorial Office at: JJBS@hu.edu.jo. After submission, a manuscript number will be communicated to the corresponding author within 48 hours.

Peer-review Process

It is requested to submit, with the manuscript, the names, addresses and e-mail addresses of at least 4 potential reviewers. It is the sole right of the editor to decide whether or not the suggested reviewers to be used. The reviewers' comments will be sent to authors within 6-8 weeks after submission. Manuscripts and figures for review will not be returned to authors whether the editorial decision is to accept, revise, or reject. All Case Reports and Short Communication must include at least one table and/ or one figure.

Preparation of Manuscript

The manuscript should be written in English with simple lay out. The text should be prepared in single column format. Bold face, italics, subscripts, superscripts etc. can be used. Pages should be numbered consecutively, beginning with the title page and continuing through the last page of typewritten material.

The text can be divided into numbered sections with brief headings. Starting from introduction with section 1. Subsections should be numbered (for example 2.1 (then 2.1.1, 2.1.2, 2.2, etc.), up to three levels. Manuscripts in general should be organized in the following manner:

Title Page

The title page should contain a brief title, correct first name, middle initial and family name of each author and name and address of the department(s) and institution(s) from where the research was carried out for each author. The title should be without any abbreviations and it should enlighten the contents of the paper. All affiliations should be provided with a lower-case superscript number just after the author's name and in front of the appropriate address.
The name of the corresponding author should be indicated along with telephone and fax numbers (with country and area code) along with full postal address and e-mail address.

Abstract

The abstract should be concise and informative. It should not exceed **350 words** in length for full manuscript and Review article and **150 words** in case of Case Report and/ or Short Communication. It should briefly describe the purpose of the work, techniques and methods used, major findings with important data and conclusions. No references should be cited in this part. Generally non-standard abbreviations should not be used, if necessary they should be clearly defined in the abstract, at first use.

Keywords

Immediately after the abstract, **about 4-8 keywords** should be given. Use of abbreviations should be avoided, only standard abbreviations, well known in the established area may be used, if appropriate. These keywords will be used for indexing.

Abbreviations

Non-standard abbreviations should be listed and full form of each abbreviation should be given in parentheses at first use in the text.

Introduction

Provide a factual background, clearly defined problem, proposed solution, a brief literature survey and the scope and justification of the work done.

Materials and Methods

Give adequate information to allow the experiment to be reproduced. Already published methods should be mentioned with references. Significant modifications of published methods and new methods should be described in detail. Capitalize trade names and include the manufacturer's name and address. Subheading should be used.

Results

Results should be clearly described in a concise manner. Results for different parameters should be described under subheadings or in separate paragraph. Results should be explained, but largely without referring to the literature. Table or figure numbers should be mentioned in parentheses for better understanding.

Discussion

The discussion should not repeat the results, but provide detailed interpretation of data. This should interpret the significance of the findings of the work. Citations should be given in support of the findings. The results and discussion part can also be described as separate, if appropriate. The Results and Discussion sections can include subheadings, and when appropriate, both sections can be combined

Conclusions

This should briefly state the major findings of the study.

Acknowledgment

A brief acknowledgment section may be given after the conclusion section just before the references. The acknowledgment of people who provided assistance in manuscript preparation, funding for research, etc. should be listed in this section.

Tables and Figures

Tables and figures should be presented as per their appearance in the text. It is suggested that the discussion about the tables and figures should appear in the text before the appearance of the respective tables and figures. No tables or figures should be given without discussion or reference inside the text.

Tables should be explanatory enough to be understandable without any text reference. Double spacing should be maintained throughout the table, including table headings and footnotes. Table headings should be placed above the table. Footnotes should be placed below the table with superscript lowercase letters. Each table should be on a separate page, numbered consecutively in Arabic numerals.

Each figure should have a caption. The caption should be concise and typed separately, not on the figure area. Figures should be self-explanatory. Information presented in the figure should not be repeated in the table. All symbols and abbreviations used in the illustrations should be defined clearly. Figure legends should be given below the figures.

References

References should be listed alphabetically at the end of the manuscript. Every reference referred in the text must be also present in the reference list and vice versa. In the text, a reference identified by means of an author's name should be followed by the year of publication in parentheses (e.g.(Brown,2009)). For two authors, both authors' names followed by the year of publication (e.g.(Nelson and Brown, 2007)). When there are more than two authors, only the first author's name followed by "*et al*." and the year of publication (e.g. (Abu-Elteen *et al*., 2010)). When two or more works of an author has been published during the same year, the reference should be identified by the letters "a", "b", "c", etc., placed after the year of publication. This should be followed both in the text and reference list. e.g., Hilly, (2002a, 2002b); Hilly, and Nelson, (2004). Articles in preparation or submitted for publication, unpublished observations, personal communications, etc. should not be included in the reference list but should only be mentioned in the article text (e.g., Shtyawy,A., University of Jordan, personal communication). Journal titles should be abbreviated according to the system adopted in Biological Abstract and Index Medicus, if not included in Biological Abstract or Index Medicus journal title should be given in full. The author is responsible for the sccuracy and completeness of the references and for their correct textual citation. Failure to do so may result in the paper being withdraw from the evaluation process. Example of correct reference form is given as follows:-

Reference to a journal publication:

Ogunseitan OA. 1998. Protein method for investigating mercuric reductase gene expression in aquatic environments. *Appl Environ Microbiol.*, **64 (2)**: 695-702.

Govindaraj S and Ranjithakumari B D. 2013. Composition and larvicidal activity of *Artemisia vulgaris* L. stem essential oil against *Aedes aegypti*. *Jordan J Biol Sci*., **6(1):** 11-16.

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

Reference to a book:

Brown WY and White SR.1985. **The Elements of Style**, third ed. MacMillan, New York.

Reference to a chapter in an edited book:

Mettam GR and Adams LB. 2010. How to prepare an electronic version of your article. In: Jones BS and Smith RZ (Eds.), **Introduction to the Electronic Age**. Kluwer Academic Publishers, Netherlands, pp. 281–304.

Conferences and Meetings:

Embabi NS. 1990. Environmental aspects of distribution of mangrove in the United Arab Emirates. Proceedings of the First ASWAS Conference. University of the United Arab Emirates. Al-Ain, United Arab Emirates.

Theses and Dissertations:

El-Labadi SN. 2002. Intestinal digenetic trematodes of some marine fishes from the Gulf of Aqaba. MSc dissertation, The Hashemite University, Zarqa, Jordan.

Nomenclature and Units

Internationally accepted rules and the international system of units (SI) should be used. If other units are mentioned, please give their equivalent in SI.

For biological nomenclature, the conventions of the *International Code of Botanical Nomenclature, the International Code of Nomenclature of Bacteria,* and the *International Code of Zoological Nomenclature* should be followed.

Scientific names of all biological creatures (crops, plants, insects, birds, mammals, etc.) should be mentioned in parentheses at first use of their English term.

Chemical nomenclature, as laid down in the *International Union of Pure and Applied Chemistry* and the official recommendations of the *IUPAC-IUB Combined Commission on Biochemical Nomenclature* should be followed. All biocides and other organic compounds must be identified by their Geneva names when first used in the text. Active ingredients of all formulations should be likewise identified.

Math formulae

All equations referred to in the text should be numbered serially at the right-hand side in parentheses. Meaning of all symbols should be given immediately after the equation at first use. Instead of root signs fractional powers should be used. Subscripts and superscripts should be presented clearly. Variables should be presented in italics. Greek letters and non-Roman symbols should be described in the margin at their first use.

To avoid any misunderstanding zero (0) and the letter O, and one (1) and the letter l should be clearly differentiated. For simple fractions use of the solidus (/) instead of a horizontal line is recommended. Levels of statistical significance such as: $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ do not require any further explanation.

Copyright

Submission of a manuscript clearly indicates that: the study has not been published before or is not under consideration for publication elsewhere (except as an abstract or as part of a published lecture or academic thesis); its publication is permitted by all authors and after accepted for publication it will not be submitted for publication anywhere else, in English or in any other language, without the written approval of the copyright-holder. The journal may consider manuscripts that are translations of articles originally published in another language. In this case, the consent of the journal in which the article was originally published must be obtained and the fact that the article has already been published must be made clear on submission and stated in the abstract. It is compulsory for the authors to ensure that no material submitted as part of a manuscript infringes existing copyrights, or the rights of a third party.

Ethical Consent

All manuscripts reporting the results of experimental investigation involving human subjects should include a statement confirming that each subject or subject's guardian obtains an informed consent, after the approval of the experimental protocol by a local human ethics committee or IRB. When reporting experiments on animals, authors should indicate whether the institutional and national guide for the care and use of laboratory animals was followed.

Plagiarism

The JJBS hold no responsibility for plagiarism. If a published paper is found later to be extensively plagiarized and is found to be a duplicate or redundant publication, a note of retraction will be published, and copies of the correspondence will be sent to the authors' head of institute.

Galley Proofs

The Editorial Office will send proofs of the manuscript to the corresponding author as an e-mail attachment for final proof reading and it will be the responsibility of the corresponding author to return the galley proof materials appropriately corrected within the stipulated time. Authors will be asked to check any typographical or minor clerical errors in the manuscript at this stage. No other major alteration in the manuscript is allowed. After publication authors can freely access the full text of the article as well as can download and print the PDF file.

Publication Charges

There are no page charges for publication in Jordan Journal of Biological Sciences, except for color illustrations.

Reprints

Twenty (20) reprints are provided to corresponding author free of charge within two weeks after the printed journal date. For orders of more reprints, a reprint order form and prices will be sent with article proofs, which should be returned directly to the Editor for processing.

Disclaimer

Articles, communication, or editorials published by JJBS represent the sole opinions of the authors. The publisher shoulders no responsibility or liability what so ever for the use or misuse of the information published by JJBS.

Indexing

JJBS is indexed and abstracted by:

DOAJ (Directory of Open Access Journals
Google Scholar
Journal Seek
HINARI
Index Copernicus
NDL Japanese Periodicals Index
SCIRUS
OAJSE
ISC (Islamic World Science Citation Center)
Directory of Research Journal Indexing (DRJI)

CABI
EBSCO
CAS (Chemical Abstract Service)
ETH- Citations
Open J-Gat
SCImago
Zoological Records
Scopus
AGORA (United Nation's FAO database)
SHERPA/RoMEO (UK)
International Institute of Organized Research (I2OR) Database

المجلة الأردنية للعلوم الحياتية
Jordan Journal of Biological Sciences (JJBS)
http://jjbs.hu.edu.jo

The Hashemite University

Deanship of Scientific Research and Graduate Studies

TRANSFER OF COPYRIGHT AGREEMENT

Journal publishers and authors share a common interest in the protection of copyright: authors principally because they want their creative works to be protected from plagiarism and other unlawful uses, publishers because they need to protect their work and investment in the production, marketing and distribution of the published version of the article. In order to do so effectively, publishers request a formal written transfer of copyright from the author(s) for each article published. Publishers and authors are also concerned that the integrity of the official record of publication of an article (once refereed and published) be maintained, and in order to protect that reference value and validation process, we ask that authors recognize that distribution (including through the Internet/WWW or other on-line means) of the authoritative version of the article as published is best administered by the Publisher.

To avoid any delay in the publication of your article, please read the terms of this agreement, sign in the space provided and return the complete form to us at the address below as quickly as possible.

Article entitled:----------

Corresponding author: ----------

To be published in the journal: Jordan Journal of Biological Sciences (JJBS)

I hereby assign to the Hashemite University the copyright in the manuscript identified above and any supplemental tables, illustrations or other information submitted therewith (the "article") in all forms and media (whether now known or hereafter developed), throughout the world, in all languages, for the full term of copyright and all extensions and renewals thereof, effective when and if the article is accepted for publication. This transfer includes the right to adapt the presentation of the article for use in conjunction with computer systems and programs, including reproduction or publication in machine-readable form and incorporation in electronic retrieval systems.
Authors retain or are hereby granted (without the need to obtain further permission) rights to use the article for traditional scholarship communications, for teaching, and for distribution within their institution.

☐ **I am the sole author of the manuscript**

☐ **I am signing on behalf of all co-authors of the manuscript**

☐ **The article is a 'work made for hire' and I am signing as an authorized representative of the employing company/institution**

Please mark one or more of the above boxes (as appropriate) and then sign and date the document in black ink.

Signed:_____ Name printed: _____
Title and Company (if employer representative) : _____
Date: _____

Data Protection: By submitting this form you are consenting that the personal information provided herein may be used by the Hashemite University and its affiliated institutions worldwide to contact you concerning the publishing of your article.

Please return the completed and signed original of this form by mail or fax, or a scanned copy of the signed original by e-mail, retaining a copy for your files, to:

Hashemite University
Zarqa 13115 Jordan
Fax: +962 5 3903338
Email: jjbs@hu.edu.jo

EDITORIAL PREFACE

Jordan Journal of Biological Sciences (JJBS) aims to publish high quality manuscripts and provide readers worldwide with high quality peer-reviewed scholarly articles on a wide variety of biological sciences such as Cell biology, developmental biology, structural biology, microbiology, entomology, toxicology, molecular biology & genetics, biochemistry, biotechnology, biodiversity, ecology, marine biology, plant biology, animal biology, physiology, and bioinformatics. JJBS is a refereed, peer reviewed quarterly international journal financed by the Scientific Research Support Fund, Ministry of Higher Education and Scientific Research in cooperation with the Hashemite University, Jordan. JJBS has been indexed by SCOPUS; CABI's Full-Text Repository, EBSCO, Science Citation Index- Zoological Abstract and recently has been included in the UGC India approved journals. JJBS is currently under evaluation to be indexed in Thomson Reuters, National Library of Medicine's MEDLINE\ Pub Med system and others.

A group of highly valuable scholars have agreed to serve on the editorial board and this places JJBS in a position of most authoritative on biological sciences. I am honored to have five associate editors: Al-Hindi (Islamic University of Gaza, Palestine.), Al-Homida, (King Saud University, Saudi Arabia), Kachani, (Western University of Health Sciences, USA), Fass, (Oman Medical College, Sultanate of Oman), and Gammoh (The University of Edinburgh). I am also delighted with our group of international advisory board members consisting from 15 countries worldwide. With our editorial board's cumulative experience in various fields of biological sciences, this journal brings a substantial representation of biological sciences in different disciplines. Without the service and dedication of our editorial board, JJBS would have never existed.

In the coming year, it is my vision to have JJBS publish a combination of manuscripts documenting rigorous studies in the area of biological sciences, and one or more manuscripts from distinguished scholar on recent advances in molecular biology. As you read throughout this volume of JJBS, I would like to remind you that the success of our journal depends directly on the number of quality articles submitted for review. Accordingly, I would like to request your participation and colleagues by submitting quality manuscripts for review. One of the great benefits we can provide to our prospective authors, regardless of acceptance of their manuscripts or not, is the feedback of our review process. JJBS provides authors with high quality, helpful reviews to improve their manuscripts.

Moreover, and as always, my thanks are also extended to the Hashemite University and Jordanian Scientific Research Support Fund for their continuous financial and administrative support to JJBS. I would like to highlight and proudly thank the group of authoritative reviewers, both local and international, who have done an outstanding work. We are honored to have you on our review list and many thanks for your valuable mentorship and contributions you provided to authors. Indeed, we count on your excellent reviews to include only high quality articles worthy of publication in JJBS. Together, we strive to make JJBS reach a remarkable rank among other international journals. I very much appreciate your support to make JJBS one of the most authoritative journals in biological sciences.

June 2018

Prof. Khaled H. Abu-Elteen
Editor-in-Chief
The Hashemite University
Zarqa, Jordan

JJBS

Volume 11, Number 2, June .2018
ISSN 1995-6673

Jordan Journal of Biological Sciences

CONTENTS

Original Articles

JJBS

Volume 11, Number 2,June 2018
ISSN 1995-6673
Pages 117 - 121

Jordan Journal of Biological Sciences

The Chemical Composition and the Antifungal Activity of the Essential Oil of *Origanum glandulosum* against *Neofusicoccum parvum*

Ammad Faiza[1,2,*] , Aouich Ahmed [2] and Boutechent Redha[2]

[1]Laboratoire de Protection et de Valorisation des Ressources Agrobiologiques (LPVRA), Département de Biotechnologie, Faculté des sciences de la nature et de la vie ; Université Blida1, BP 270 Blida 09000, Algérie.
[2] Faculté des sciences de la nature et de la vie, Département de Biotechnologie, Université Blida1, BP 270 Blida 09000, Algérie.

Received July 19, 2017; Revised September 26, 2017; Accepted October 19, 2017

Abstract:

The essential oil (EO) extracted from the leaves and flower of the *Origanum glandulosum* was screened for its *in vitro* antifungal properties. Antifungal activity (AA) was determined using a disc diffusion method against *Neofusicoccum parvu*m (KF465685) phytopathogenic fungus attacking grapevine wood. The essential oil exhibited high reducing power (IM: 41%). These results suggest that the aerial part of *O. glandulosum* have significant antifungal activities. The chemical composition of essential oils isolated by hydro-distillation and analyzed by Gas Chromatography/mass spectrometry (GC-MS) showed presence of volatile compounds, representing 99.96% of the total oil. The oil was characterized by relatively-high amounts of monoterpene hydrocarbons, phenolic monoterpenes, and sesquiterpenes. The principal components identified included: carvacrol (48.42%), ã–terpinene (27.09%), Para-cymene (16.9%) and β-caryophyllene (3.45%).The results of this study showed that the toxicity of the oregano oil varies with increasing the applied doses on the one hand, and a relatively gradual efficiency versus time, which resulted in improved efficiency on the other hand. This is the first study of its kind on the use of the essential oils of *oregano* to control this tested fungus.

Keywords: Antifungal activity, Essential oil, *Neofusicoccum parvum*, *Origanum glandulosum.*

1. Introduction

The genus *Oregano* (*lamiaceae*) is distributed throughout the world; most of which are native to the eastern part of the Mediterranean area, Europe, Asia, and North Africa. Letswaart (1980) described forty-nine species belonging to ten different sections. The most popular include: *O. vulgare*, *O. floribodum*, *O. marjona*, *O. dictamus*, *O. glandulosum,* and *O. scabrum*. A crucial part of the flora of Algeria, the genus *Origanum* includes four main species, among these is the *O. glandulosum,* which is an endemic spontaneous plant growing in North Africa [Algeria and Tunisia] (Ben Hamida and Abdelkéfi, 2001). It is an herbaceous plant characterized by a pleasant flavour, and is largely used in traditional medicine for its sedative, antispasmodic, expectorant, and carminative properties. In addition, oregano oil also showed antibacterial, antifungal, antiparasitic, antimicrobial, and antioxidant properties.

Essential oils are volatile, natural, complex compounds characterized by a strong odor produced by plants as secondary metabolites. The essential oil composition is strongly influenced by intrinsic factors such as species, cultivar, clone, ecotype, and ecological factors including the geographical origin, climatic and soil conditions, biotic and technological factors, cultivation techniques, storage conditions of raw materials and processing technologies (Russo *et al*., 2012). In this study, the extraction of the essential oil of *O. glandulosum* was done through the hydro-distillation method. The chemical composition of the oil was analyzed by Gas Chromatography/mass spectrometry (GC-MS). Then an antifungal investigation against *N. parvum* was performed. This type of fungus colonizes wood tissues and causes Dieback. It is also among the responsible agents causing Black Dead Arm (BDA), one of the fatal diseases of arboreal, viticultural and forestry heritages. BDA caused by the *Botryosphaeria* family, eutypiosis and esca are the most destructive diseases causing decline and loss of the productivity of vineyards in world (Úrbez-Torres, 2011) and pose a real threat to the sustainability of vineyards, especially young ones. This has become a serious problem in most vine-growing regions. Algeria is among those regions affected by these diseases as confirmed in (Ammad *et al.*, 2014 a; 2014. b).

It should be noted that no chemical control of these wood diseases has been provided since the prohibition of the synthetic chemical, sodium arsenite, in 2001, because of its carcinogenicity, high and acute residual toxicity, and

* Corresponding author. e-mail: sahraoui_a_f@yahoo.fr.

other side effects on humans (Lingk, 1991; Unnikrishnan and Nath, 2002). For these reasons, prophylactic methods are mainly recommended in order to limit the development of those phytopathogenic fungi. Several studies have reported the antifungal effectiveness of the essential oils of some medicinal plants (Jayasena and Jo, 2013).These natural products have the potential to be safe fungicides to replace the synthetic ones; they are biodegradable in nature, non-pollutant and possess no residual or phytotoxic properties. Therefore, this study aims to investigate, for the first time, *in vitro* antifungal activities (AA) of *Origanum glandulosum* EO in the treatment of phytopathogenic fungi attacking the wood of grapevine trees. This study identifies the EO composition by GC/MS after being extracted by Hydro-distillation from the aerial parts of oregano. Results show that EO exerts a significant AA against studied fungi attacking the wood of the grapevine and can be also effective to control the fungal diseases in the agriculture sphere.

2. Materials and Methods

2.1. Plant Material

The leaves and flowers of *Origanum glandulosum* used for this study were collected during 2012 from Larabaa (Blida), located in the north of Algeria (36°33' 55''N, 3° 09' 14''). Botanical identification of this species was authenticated by using a determination key (Quezel and Santa, 1963).The plant material used for the extraction of the essential oil was air-dried in free air.

2.2. Extraction of the essential oils

The essential oils were extracted by the Hydro-distillation of dried plant materials (100 g of leaves and flowers in 500 mL of distilled water) using a Clevenger-type for 5 h. The Bottles of oil were covered with aluminum paper to protect them against any negative effects of light, and were stored in a refrigerator at a temperature of 4 ° C. The actual yield based on the dried weight of the sample was calculated.

2.3. Analysis of the essential oils

2.3.1. Gas chromatography–mass spectrometry analysis (GC-MS)

Chromatographic analysis was performed using a Gas Chromatography (GC) to separate the complex mixtures of the volatiles identified and quantified in a relatively short time (Sharp,1986). GC-MS was performed using a Perkin-Elmer Clarus 600 mass spectrometer with a silica capillary column of 50 m length, and 0.22 mm inner diameter of 50 µm film- thickness. Chromatogram was recorded with temperature ramp in a four-minute step at 40 °C then at a further increase of the temperature up to 250°C at a rate of 30°C/ min. Helium was used as the carrier gas at a rate of 1 mL/min. The oil sample (0.1 µL) was introduced directly into the source of the MS (mass spectrometry) via a transfer line (280° C) with a split ratio of 1:50. EO components were identified based on their retention indices (determined with reference), and were calculated using Biot's law equation 1 as follows (Eqn. 1):

$$[\alpha] = \frac{\alpha}{C.l} \quad \text{(Eqn. 1)}$$

Where [α] is the specific rotatory power, (l) is the optical path length of the tank; α is the optical rotation; and C is the concentration of the solution in g/mL. The EO components were identified on the basis of their retention indices (determined with reference to a homologous series of normal alkanes), and also after comparing their masses, obtained by the different fragmentation patterns of the mass spectroscopic analysis, with the masses reported in the related literature (Adams, 2007).

2.4. Fungal material

One strain of the fungal material (*Neofusicoccum parvum*) meant for evaluating the effectiveness of the treatments using the tested essential oil was obtained by a personal collection. *Neofusicoccum parvum* was isolated from the infected wood of a grapevine (Ammad et *al.,* 2014 b), and was identified using a combination of morphological and cultural characters confirmed by molecular analysis, Internal transcribed spacer (ITS) and (β-tubuline primer). Culture of the fungi was maintained on potato dextrose agar (PDA) and was stored at 4 °C.

2.5. Antifungal activity assays

The effects of volatile essential oils were assayed by inoculating mycelia plugs in the center of a PDA Petri dish. The essential oil (EO) was dissolved in tween water solution (3%) and three doses of this essential oil were prepared (0.25, 0.50 and 0.75%). Sterile filter paper discs (7cm diameter), soaked in 30 µL of each dilution of the EO, were placed on the inner surface of the Petri-dish lid (Inouye *et al.*, 2006).The dishes were sealed with Para film and incubated upside-down at 25˚C. Measurements of colony radius (in cm) were made after five days. Three replicates per treatment were carried out, and each experiment was repeated at least twice. Data were expressed as percentage inhibition of mycelia growth, according to the formula of Pandey *et al*, (1982). Using the following formula: (P Ig = (DT-D)/DT) x 100), where P Ig is the percentage of inhibition growth, DT is the mean diameter of mycelial growth in control, and D is the mean diameter of mycelial growth in treatment. The estimation of mycelia growth was carried for ten days; three days following the treatment with the essential oil. For a better measure of the diameters of the mycelia growth, digital pictures of all plates were taken and treated with Image Tool software (3.1), three (03) measures were selected for each diameter.

2.6. Statistical analysis

The results of antifungal potency were treated with Excel and SYSTAT software (ver.12), SPSS 2009. The hypothesis of the antifungal efficacy of the essential oil was tested by the analysis of variance with the Global Linear Model (GLM).

3. Results

3.1. Chemical composition

The oil yield obtained through the Hydro-distillation of the *O. glandulosum* leaves and flowers was 0.98 %. The major components, representing 99.96% of the essential oil were identified by the GC technique. The main

components are presented in Table 1. The chemical composition of the *O. glandulosum* oil was dominated by monoterpene hydrocarbons fraction with a predominance of phenolic compounds and sesquiterpenes. Similar to the essential oil extracted from other *Origanum* species, this oil was characterized by high percentages of phenols; the major compounds of the oil were (carvacrol or its isomer the thymol) (48.56%). This oil can be classified as a carvacrol chemotype.

Table 1 . Principal chemical composition, retention time and percentage composition of the essential oil of *O. glandulosum* collected from Larabaa,

Pick	Compound	Formula	Réel Time (min)	Retention time	Percentage (%)
1	Carvacrol (Isothymol)	$C_{10}H_{14}O$	9.63	33.40	48.42
2	γ - Terpinene	$C_{10}H_{16}$	10.62	16.55	27.09
3	Para-Cymene	$C_{10}H_{14}$	17.10	14.44	16.01
4	β-Caryophyllene	$C_{15}H_{24}$	17.35	39.43	3.45
5	α-Terpinene	$C_{10}H_{16}$	9.33	13.91	2.55
6	Myrcene	$C_{10}H_{16}$	15.77	12.61	2.44

3.2. Antifungal activity of essential oils

The antifungal activities (AA) recorded in this study, which represents the inhibition of radial growth on solid medium, reveals that the EO of *O. glandulosum* possesses potential AA against *N. parvum* fungi. The effects of the EO dose with different concentrations are summarized in Table 2. The statistical analysis of variance revealed significant results (Figure. 1); all the tested concentrations inhibit the growth of fungus at all concentrations. At a concentration of 0.25%, *O. glandulosum* showed low toxicity at the beginning of its application to an average toxicity at the end of treatment. On the one hand, the two concentrations D3 (0.75%) and D2 (0.50%) showed more inhibitory effect compared to D1 (0.25%). On the other hand, no inhibition was registered even after ten days with the control (Figure 1 a). The different periods showed significant probability (Figure 1 b).

The results obtained in this section (AA) indicate that the volatile oils exhibited different degrees of inhibition on the growth of the tested fungi, the higher concentrations inhibited more efficiently than the diluted ones, and the duration of treatments showed interested effectiveness.

Table 2 . Antifungal Activity of the Essenti al Oil of *O. glandulosum.*

Fungi	Dilution/ time	D1 (0.25%)	D2 (0.50%)	D3 (0.75%)	Control
N.parvum	T3 /Days(05)	17.20	12.25	11.24	18.4
	T2/Days(10)	15,2	10.35	9,07	23.14
	T1/Days(15)	38.12	30.00	28	54.21

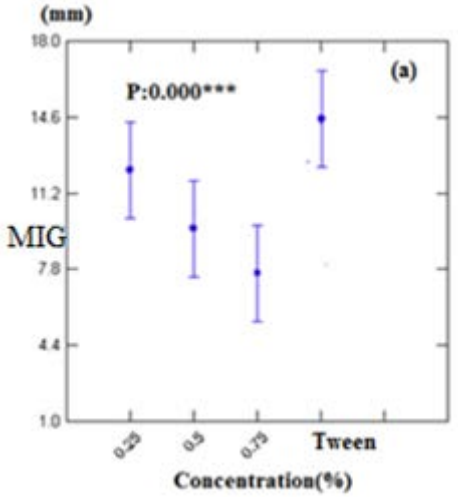


Figure1a. Variance Analysis (GLM) global linear model of time on the efficacy of essential oil against the pathogenic fungi MIG: Mycelia inhibition growth (mm), T1: 03 days, T2: 05 days and T3: 10 days P: probability , N.S.: non significant, * : significant Probability at 5 % ; ** : significant Probability 1 % ; *** : significant Probability at 0,1 %

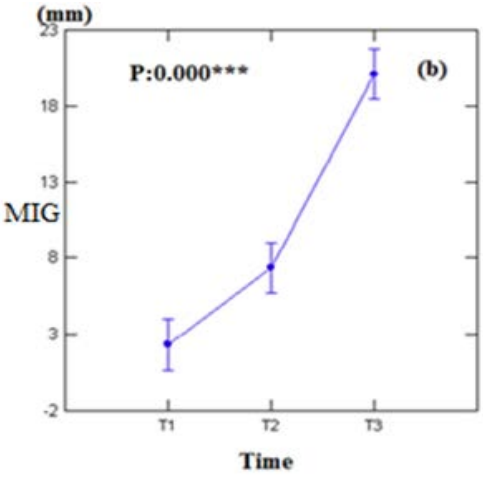


Figure1b. Variance Analysis (GLM) of dose on the efficacy of essential oil against the pathogenic fungi MG: Mycelia growth (mm), D1: 0.25% ,D2: 0.50%, D3: 0.75% s and D1: 1%, control: Dimethylsulfoxyde) solution (3%)(DMSO), P:probability , N.S.: nonsignificant, * : significant Probability at 5 % ; ** : significant Probability 1 % ; *** : significant Probability at 0,1 %

4. Discussion

Black Dead Arm (BDA), a disease of grapevines caused by *N. parvum,* can be a real nuisance to the economy. The absence of an effective treatment against these funguses has become a real problem**.** The present study is an evaluation of a treatment method which uses oregano as a protective safe chemical against *N. parvum***.**

In this study, the chemical results indicated that *O. glandulosum* EO was characterized by the high proportion of monoterpenes, notably cavracrol, which was found to possess a good biological activity against *E. coli* and *Bacillus subtilis*. This component has proven effective in bacterial membrane perturbations that lead to leakage of intracellular ATP and potassium ions and ultimately cell death (Pinto *et al*.,2006).

The essential oil of oregano has been investigated in earlier studies, which showed that carvacrol is the major component of the oil, but in different levels. Similar results by Bejaoui *et al.* (2013), concerning many essential oils extracted from *O. glandulosum* grown in some regions of Tunisia showed that the chemical composition is dominated by monoterpenes and that the highest proportion of carvacrol (68-83%) can be obtained from the oregano plant. The percentages of the components obtained in this study were different from other results concerning the essential oil of *O. glandulosum* reported by Ouled Lyiche and Djebel Megriss (Sétif) (East region in Algeria) (Ruberto *et al*, 2002). According to Maarse, (1974) and Bousbia (2004), these differences in the oil composition and yield can be attributed to several factors, including climatic and geographic conditions, time of collection, and extraction methods. According to Vaughn and Spencer, (1991) as well as Panizzi *et al*, (1993) and Caccioni and Guizzardi, (1994) the essential oils produced by different plant species belonging to the Meliaceae, Rutaceae, Asteraceae, Lamiaceae, Abiateae, and Canellaceae families are in many cases biologically active and have antimicrobial, allelopathic, antioxidant and bioregulatory properties. The antimicrobial properties can be related to the presence of active constituents, mainly attributed to isoprene's such as monoterpenes and sesquiterpenes and other hydrocarbons.

Apparently, these essential oils with their high phenolic are more effective, and have a broad spectrum of activity against filamentous fungi and insects (Cosentino, 1999). According to Dinan *et al*, (2001), the plant secondary compounds possess several modes of action against the fungal strain, but in general, their action takes place in three phases: the attack of the wall by the plant extraction, resulting in an increase permeability and losing of cellular constituents, the acidification of the inside of the cell blocking the production of cellular energy and synthesis of structural components, and finally the destruction of the genetic material leading to the death of fungi.

Several authors have showed that phenols were not the only compounds responsible for the activity. All substances of the chemical composition should be taken into account. In this regard, (Cosentino, 1999). Lahlou, (2004) and Klaric *et al*., (2006) reported that the activity of the essential oil is higher than that of the majority of its composition tested separately. The antifungal activity of the essential oil tested in this study was attributed to the presence of phenol, sesquiterpenes and monoterpenes, and the synergism between components which all play an important role. The chemical structure of the constituents of the EOs directly influences their activity (Guinoiseau, 2010).

5. Conclusions

In this study, *O. glandulosum* (EO) led to the growth inhibition of *N. parvum*. All tested concentrations were found to be lethal under the test conditions. Based on the present study, it could be concluded that EO of *O. glandulosum* possesses fungi toxic activities that can inhibit the growth of phytopathogenic fungi. Moreover, it was found that EO was characterized by a relatively-high content of phenol, showing a high yield of oils rich in carvacrol, p-cymene, and c-terpinene, which are known to possess an important antifungal activity. The data presented confirm the antifungal potential of the *O. glandulosum* essential oil. The EO tested represents an inexpensive source of natural antifungal substances for use in pathogenic systems. It would be beneficial to test the effectiveness of each component of this essential oil separately to be able to confirm the source of the oil's efficacy i.e. whether its effectiveness is related to all the substances in the oil or specific components. It is really significant to assess the morphological alterations and the modes of action of this oil on phytopathogenic fungi in further studies.

Acknowledgements

This study was conducted in the Laboratory of Phytopathology of the Department of Biotechnology in the University Blida 1, Bilda, Algeria. We send them our regards. We also would like to express our gratitude to the pharmaceutical company, SAIDAL Médea, Algeria.

References

Adams RP. 2007. **Identification of Essential Oil Components by Gas Chromatography/Mass Spectrometry**, Allured Publishing Co. Carol Stream, Illinois, 4th Ed.

Ammad F, Benchabane M and Toumi M. 2014a. Diversity of fungal trunk pathogens associated with grapevine dieback of grapevine in Algeria. *Jordan J Biol Sci.,* **7(1):** 35 -39.

Ammad F, Benchabane M, Toumi M, Belkacem N, Guesmi A, Cherif A, Lecomte P and ,Merah O.2014b. Occurrence of *Botryosphaeria* species associated with grapevine dieback in Algeria. *Turk J Agric Fores* ., **38**: 865-876.

BenHamida-Ben Azzedine, N. and Abdelkéfi, M.M. 2001. Antibacterial screening of *Origanum marjorana* L. oil from Tunisia. *J. Essent. Oil Res*., **13**: 295-297.

Bejaoui A, Chaabane H, Jemli M, Boulila A and Boussaid M. 2013. Essential oil composition and antibacterial activity of *Origanum vulgare* subsp. *glandulosum* Desf at different phenological stages. *J. Med. Food*., **16**: 1115–1120.

Bousbia N. 2004. Extraction et Identification de Quelques Huiles Essentielles (Nigelle. Coriandre, Origan, Tym, Romarin) : Etude de leurs activités antimicrobiennes- Thèse magistère ; INA-Alger ; pp 130.2004.

Caccioni D.R.L. and Guizzardi M. 1994. Inhibition of germination and growth of fruit and vegetable post-harvest pathogenic fungi by essential oil components. *J. Essential. Oil Research* ., **6**: 173-179.

Cosentino S. 1999 . *In vitro* antimicrobial activity and chemical composition of Sardinian *Thymus* essential oils. *Lett. Appl. Microbiol.*, **29:** 130-135.

Dinan L, Savchenko T and Whiting P. 2001. On the distribution of phytoecdysteroids in plants. *Cellular and Molecular Life Sci.,* **58 (8):** 1121-1132.

Guinoiseau E. 2010. Molécules, antibactérienne issues d'huiles essentielles: séparation, identification et mode d'action. *Thèse de Doctorat*, Univ. Corse; Option: Biochimie- Biologie moléculaire ; France ; P50.

Inouye S, Uchida K, Maruyama N, Yamaguchi H and Abe S. 2006. A novel method to estimate the contribution of the vaporactivity of the essential oil in agar diffusion assay. *J. Med. Mycol* ., **47** : 91-98.

Inouye S, Yamaguchi H_and Takizawa T. 2001. Screening of the antibacterial effect of a variety of essential oils on respiratory tract pathogens, using a modified dilution assay method, *J. Infect. Chemother*., **7(4):** 251-254.

Jayasena D D and Jo C. 2013. Essential oils as potential antimicrobial agents in meats and meat products: A review. *Trends Food Sci. Tech*., **34**: 96–108.

Klaric M.S, Kosalec I, Mastelic K J, Pieckova E and Pepeljnak S. 2000. Antifungal activity of thyme (*Thymus vulgaris* L.) essential oil and thymol against moulds from damp dwellings. *Lett Appl Microbiol*., **44**: 36–42.

Lahlou M. 2004. Method to study phytochemistry and bioactivity of essential oil. *Phytotherapy Res.,* **18**:435-448.

Letswaart J.H.A. 1980. **Taxonomic Revision of the Genus *Origanum***, Leiden Botanical Series, Vol.4. Leiden University Press : The Hague, The Netherland.

Lingk W. 1991. Health risk evaluation of pesticides contaminations in drinking water. *Gesunde Pflanzen* **., 43**: 21-25.

Maarse H. 1974 . Volatile oil of *Origanum vulgare* L. ssp. *vulgare*. III. Changes in composition during maturation. *Flavour Ind.,* **4**:278-281.

Pandey DK, Tripathi NN, Tripathi RD and Dixit SN. 1982. Fungitoxic and phytotoxic properties of the essentiel oil of *Caesulia axillaris Roxb.* (Compositae). *Angerwandte Botanik*., **56**: 256-257.

Panizzi L, Flamini G, Cioni, PL and Morelli I. 1993. Composition and antimicrobial properties of essential oils of four Mediterranean Lamiaceaes. *J. Ethnopharmacol.* **39**:167-170.

Pinto E, Pina-Vaz C, Salgueiro L, Jose Goncalves M, Costa-de-Oliveira S, Cavaleiro C, Palmeira I, Rodrigues I and Martinez-de-Oliveira J. 2006. Antifungal activity of the essential oil of *Thymus pulegioides* on *Candida*, *Aspergillus* and dermatophyte species. *J Medical and Microbial***., 55**: 1367-1373.

Ruberto G, Baratta MT, Sari M. and Kaâbeche M. 2002. Chemical composition and antioxidant activity of essential oils from Algerian *Origanum glandulosum* Desf. *Flavour Fragr. J*.; **17**: 251-254.

Russo M, Galletti G.C, Bocchini P, Carnacini A. 1988. Essential oil chemical composition of wild populations of Italian oregano spice (*Origanum vulgare* ssp. *hirtum*(Link) Ietswaart): A preliminary evaluation of their use in chemotaxonomy by cluster analysis. 1. Inflorescences. *J Agric Food Chem.*, **46**: 3741-3746.

Sharp M.E. 1986. Evaluation of a screening procedure for basic and neutral drugs. N-Butyl, chloride extraction and megabore capillary Gas chromatograph. *Sci. J*., **19**:83-100.

Quezel P and Santa S. 1963 . Nouvelle flore de l'Algérie et des régions désertiques méridionales : tome I.PARIS. 558 P.

Unnikrishnan V and Nath B.S. 2002. Hazardous chemicals in foods. *Indian J Dairy Biosci*., **11**: 155–158.

Úrbez-Torres JR. 2011. The status of *Botryosphaeria* species infecting grapevines. *Phytopathologia Mediterranea*., 50: S5-S45.

Vaughn S.F and Spencer G.F. 1991 . Volatile monoterpenes inhibit potato tubersprouting. *Potato J.,* **68**: 821-831.

JJBS

Volume 11, Number 2,June 2018
ISSN 1995-6673
Pages 123 - 128

Jordan Journal of Biological Sciences

The Relationship between *Helicobacter pylori* (*H. pylori*) and Atopy and Allergic Diseases

Eman M. Albataineh[1,*], Nedal A. Alnawaiseh[2], Enas Al-Zayadneh[3], Rashed Al-amer[4], Nasser Kaplan[5] and Mohammad A. Abu-lobbad[1]

[1]*Department of Microbiology and Immunology,* [2]*Department of Public Health,* [3]*Department of Pediatrics, Faculty of Medicine, Mutah University,* [4]*Internship Doctor, Al-Karak Governmental Hospital, Al-Karak,* [5]*Department of Microbiology and Immunology, Faculty of Medicine, Jordan University of Science and Technology, Irbid, Jordan*

Received August 21, 2017; Revised October 5, 2017; Accepted October 21, 2017

Abstract

Reduced exposures to gastrointestinal microbiota have been proposed as an explanation for the increase in the prevalence of allergic diseases. The aim of this research is to study the relationship between *H. pylori* and atopy/allergic diseases. 210 children (aged 2-16 years old) with or without allergic diseases were selected from the Asthma Clinic in Al-Karak Governmental Hospital to participate in the study. Their parents were asked to fill out questionnaires. An Atopy test was done and the serum IgG antibodies to the *H. pylori* antigens were measured. Out of the 210 children, 38 were *H. pylori*-positive patients (18.1%), 31 cases (14.1 %) were atopic, and 60 patients (28.6%) were asthmatic. Out of the 38 *H. pylori* positive patients, only one case (2.6 %) was atopic. A significant negative association between *H pylori* and asthma was observed in the less than nine-year-old age group (p value = 0.007). The negative association between *H. pylori* and atopy was significant in males (p value = 0.02).The factors that best predict asthma include a family history of asthma, positive *H. pylori*, positive allergy test (p values of <0.005). Our findings showed that there is an inverse correlation between *H. pylori* and atopy.

Keywords*: Helicobacter pylori*; Asthma; Childhood Asthma, Jordan

1. Introduction

It is now known that allergic diseases develop from the interaction between the host immune system and some environmental factors (Rigoli *et al.,* 2011). The high geographical variability in the prevalence of these diseases suggests a decisive impact of the environmental factors (such as the geo-climatic and the socioeconomic ones) on their pathogenesis. Indeed, allergies are more common in the northern hemisphere, as well as in developed countries more than in developing ones (Mallol *et al.,* 2013). The characteristic epidemiological trend of atopic diseases seems to be also linked to the environmental changes that had occurred over the last decades in industrialized countries including the increase in outdoor and indoor pollution (combustion of fossil fuel, high volume of traffic, biomass combustion products, tobacco smoke), climatic changes (warmer temperature that causes early springs), and the improvement of hygienic conditions (changes in exposure to microbiota).

H. pylori, gram-negative, microaerophilic gastric bacteria persistently colonize much of the world's population. Whereas nearly all adults are *H. pylori*-positive in developing countries, with socioeconomic development, prevalence has decreased substantially (Dooley *et al.,* 1989). *H. pylori* is almost exclusively acquired in childhood (Blaser and Atherton, 2004), and the antibody responses are present for decades or for life, and are consistent with the persistent gastric colonization. *H. pylori* virulence is affected by the presence of the 35-40-kb cag pathogenicity island that can be detected by the identification of the CagA gene or its product (CagA). *H. pylori* colonization induces a continuous gastric inflammation, which is more pronounced with CagA+ strains (Suerbaum and Michetti, 2002), and leads up to a diminished gastric acidity (Kuipers *et al.,* 1995). Serologic assays to detect antibodies to the CagA protein enhance the overall detection of *H. pylori*; in particular the detection of the more interactive (CagA+) organisms.

As the hygiene hypothesis confirms, the most important factor associated with the large spreading of the atopic disease is the decreased exposure to food-born and oral-fecal infections, including the *Helicobacter pylori* (*H. pylori*) infection, as a result of improvements in the hygienic conditions in developed countries (Matsushima and Nagai, 2012). There is some evidence of an inverse association between atopy/allergic diseases and the *H.*

* Corresponding author. e-mail: emanbatayneh@yahoo.com.

pylori infection, although further studies are still needed (Reibman *et al.,* 2008; Daugule *et al.,* 2015).

Despite the fact that these clinical studies confirm the inverse association between *H. pylori* and asthma, some studies reported different results regarding asthma, other allergic diseases, and in general atopy. On the other hand, many researchers denied the hygiene hypothesis, considering gastrointestinal infections as triggers of allergic diseases, rather than protective factors because of their capability in increasing the mucosal permeability. Thereby asserting that these infections could have facilitated the penetration of allergens and the loss of oral tolerance (Jun *et al.,* 2005; Baccioglu *et al.,* 2008; Raj *et al.,* 2009; Lee *et al.*, 2015). Moreover, a recent study points that *the H. pylori* infection acts most likely as a good marker for the presence of poor hygiene (Miftahussurur *et al.*, 2017).

The aim of this article was to conduct a cross-sectional study on the relationship between *H. pylori* and atopy/allergic diseases to investigate whether or not there is negative association between *H. pylori* and atopy or allergic diseases among children in the area.

2. Patients and Methods

2.1. Study Design

The association between atopy/allergic diseases and the *H. pylori* infection is the focus of the current research investigating the *H. pylori* seroprevalence in atopic patients, those with allergic diseases, and in healthy individuals. Atopy is defined through the skin prick test reactivity to a panel of aeroallergens (olive pollen, mixed-grass pollen, dog dander, cat dander, Dermatophagoides Farinae, Dermatophagoides pteronyssinus, Cereals, Compositae, salsolakali and Wallpollitory). Allergic diseases are defined with the presence of a diagnosis of asthma, self- reported allergic rhinitis (Hey fever), self-reported atopic dermatitis, self- reported food allergy, or with the presence of allergic manifestations (such as pruritus of skin or eyes, nasal discharge, coughing and dyspnea) determined through questionnaires. The *H. pylori* infection status will be tested by serum anti *H. pylori* IgG and anti-CagA antibodies.

Several cross-sectional studies have investigated the relationship between the *H. pylori infection* and allergic diseases. Most of them have been conducted in developed countries, where atopy is more common, and the decreasing trend of the *H. pylori infection* is effective. This study will be the first of its kind to be done in Jordan. It was approved by the Ethics and Scientific Committees of the Faculty of Medicine at Mutah University. It was conducted between October, 2016 and June, 2017 at Al-Karak Governate Hospital in Al-Karak, Jordan. After signing the consent forms by the parents, 210 child patients with/without atopy or allergic diseases (cases) filled out questionnaires, and then two tests were done for all of them, namely the skin prick test (done in the Child Respiratory Clinic of Al- Karak Governmental Hospital), and the *H. pylori* test (done in a private laboratory) . The questionnaire included questions related to the demographic characteristics (age, sex, and residence), socio-economic status (education level, the monthly family income), and clinical characteristics (history of asthma, allergic rhinitis (Hey fever), atopic dermatitis, food allergy, family history of allergic diseases, and history of any *H. pylori* related- gastro-intestinal (GI) symptoms as dyspepsia regurgitation heart burn, anemia, poor growth, and any use of anti-acids). The body mass index (BMI) was calculated for all the participants.

The Odds Ratios (OR) of atopy and allergic diseases associated with the presence of *H. pylori* will be estimated. The effect of other factors such as age and sex will be analyzed as well. If the observed association is significant, more insights into the underlying mechanisms could provide clues to the possible therapeutic opportunities of allergic diseases.

2.2. Serum Antibody Analysis

Serum anti-*H. pylori* IgG antibody levels were determined in patient serum using a one-step *H pylori* test device from ABON (Herbrink and Van Doorn, 2000; Alsaimary *et al.*, 2014). Blood samples were collected from the children. The blood was either allowed to be clotted and the serum separated, or centrifuged (400xg for 5 mins.) to separate the serum. Then the serum was immediately examined by the rapid diagnostic *H. pylori* kit–Abon Biopharm (Hangzhou) Co., Ltd. P.R. China. The one- step *H. pylori* test device (serum/plasma) is a qualitative membrane based immunoassay for the detection of *H. pylori* antibodies in serum or plasma. In this test procedure, the anti-human IgG is immobilized in the test line region of the test. After the specimen was added to the specimen well of the device, it reacted with the *H. pylori* antigen coated particles in the test. This mixture migrates chromatographically along the length of the test, and interacts with immobilized anti-human IgG.

2.3. Statistical Analysis

Categorical variables were analyzed using the chi-square test or Fisher's exact test. Basically, the chi-square test was used in this study. However, Fisher's exact test was used instead of chi-square test when >20% of the expected frequencies were five or less. The binary logistic regression model was used to analyze dichotomous variables according to predictor variables. Independent variables with p values <0.20 in univariate analyses, and those already known to be strongly associated with the outcome variable were examined in multiple logistic regression models. A p-value <0.05 was considered significant. All p values are presented without correction for multiple testing. All analyses were performed with the Statistical Package for the Social Sciences, (ver. 16.0) for Windows software (SPSS Inc., Chicago, IL).

3. Results

3.1. Characteristics of the Study Groups

The youngest child examined in this study was two years old, while the oldest was sixteen years old. One-hundred and twenty-eight children (61%) were males and 82 (39 %) were females. The mean age was 8. 7 ± 3.4. Thirty eight of the cases (18.1) had a positive *H. pylori* test (male=17.9, female=18.3). Children with *H. pylori* had a mean age of 9.4 ± 3.1, while those without this infection had a mean age of 8.6 ± 3.5; no significant difference was

observed. Thirty one of the cases (14.8) had positive allergy test (Skin Prick Test). The mean age of children with atopy was 7.6 ± 4.3, while the mean age of those without atopy was 8.9 ± 3.2; no significant difference was indicated. The Mann Whitney U test was used to compare the mean age of children. Sixty of the cases (28.6%) had doctor diagnosed- asthma. The percentages of the self-reported allergic rhinitis (Hey fever), self- reported atopic dermatitis, self- reported food allergy were 48.1, 19, 26.2, respectively (Table 1).

Table 1. Characteristics of the study population.

Variable	Mean± SD	Number	Percentage (210=100%)
Weight (Kg)	38.2±1.7		
Height (cm)	134±220		
BMI	20.8±1.1		
Age (Years)	8.7±3.4		
Gender			
Male		128	61
Female		82	39
H pylori test			
Positive		38	18.1
Negative		172	81.9
Atopy			
Positive		31	14.8
Negative		179	85.2
Doctor diagnosed asthma			
Positive		60	28.6
Negative		150	71.4
Self-reported hay fever			
Positive		101	48.1
Negative		109	51.9
Self-reported eczema			
Positive		40	19
Negative		170	81
Self- reported food allergy			
Positive		55	26.2
Negative		155	73.8
Family history of asthma			
Positive		70	33.3
Negative		140	66.7
Family history of hay fever			
Positive		76	36.2
Negative		134	63.8
Family history of food allergy			
Positive		38	18.1
Negative		172	81.9
Family history of eczema			
Positive		50	23.8
Negative		160	76.2

3.2. H. pylori Status and Atopy

The skin prick test was done for all the child participants. The percentage of atopy in the whole data was 14.8. Similar findings were also obtained from an international study (Asher *et al*., 1995). The aero-allergens used in the test were olive pollen, mixed-grass pollen, dog dander, cat dander, Dermatophagoides Farinae, Dermatophagoides pteronyssinus, Cereals, Compositae, salsolakali and Wallpollitory; there sensitization percentages are presented in Figure 1.

The *H. pylori* test was done for all of the study population. The prevalence of seropositivity was 18.1.The *H. pylori*-related gastrointestinal symptoms were recorded based on the questionnaires provided. The percentages of these symptoms in both seropositive and seronegative patients were shown in Figure 2 (p value <0.05).

Out of the 38 *H. pylori*-positive patients, one case (2.6 %) was atopic, while out of the 172 *H. pylori* negative patients 30 cases (97.4%) were non-atopic, with the difference being statistically significant (p Value = 0.02) (Table 2, Figure 3).

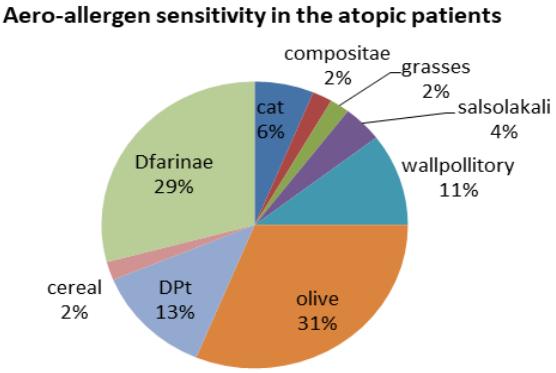


Figure 1. Percentages of aero-allergen sensitivity in the atopic patients

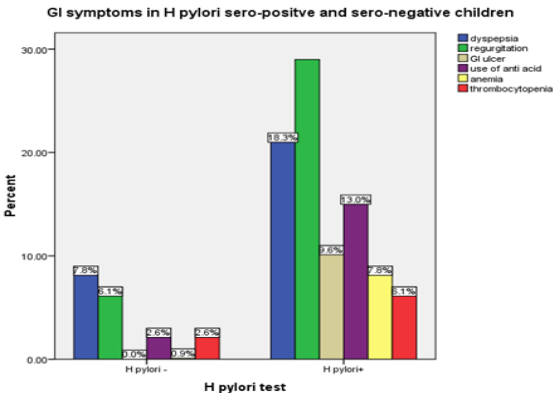


Figure 2. Prevalence of *H pylori*- related symptoms in sero-positive and sero- negative children**.**

Table 2. Frequency of distribution of allergy in the two groups of *H. pylori* positive and negative children.

		H Pylori Test		Chi-Square	P-Value
		No N (%)	Yes N (%)		
Allergy Test	No	142(82.6)	37(97.4)	5.426	0.020
	Yes	30(17.4)	1(2.6)		

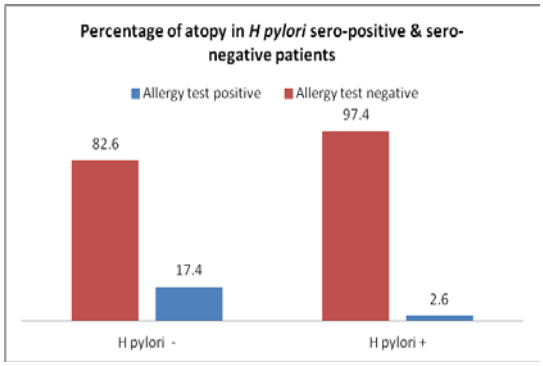


Figure 3. Percentages of aero-allergen sensitivity in the atopic patients

3.3. Triggers of H. pylori-Asthma or Atopy Inverse Association

The effects of sex and age on the negative association between *H. pylori* and atopy or asthma were studied. The obtained results showed that there is a significant negative association between *H. pylori* and doctor diagnosed asthma in the less than nine-year-old age group (*p* value= 0.007); the result for the association of *H. pylori* with atopy was not significant for this age group. Whereas the negative association between *H. pylori* and atopy was significant in males (*p* value= 0.02). (Tables 3, 4). Also, the Odd-Ratio Mantel Haenszel (ORMH) was obtained (ORMH age = 3.5, ORMH gender = 2.2).

Table 3. Frequency of distribution of children according to age and the situation of *H. pylori* and doctor diagnosed asthma.

Age group	Doctor diagnosed asthma	*H Pylori* Test No n (%)	*H Pylori* Test Yes n (%)	Chi-Square	P-Value	Mantel Haenszel Chi-(p-value)	OR	95%CI
≤ 9, y	Negative	87 (79)	12 (52.2)	7.24	0.007	8.94 (0.003)	3.467*	1.357-8.862
	Positive	23 (21)	11 (47.8)					
>9,y	Negative	44 (71)	7 (46.7)	3.18	0.074		1**	-
	Positive	18 (29)	8 (53.3)					

*risk estimate ,**reference value

Table 4. Frequency of distribution of children according to gender and the situation of *H. pylori* and atopy.

Sex	**Allergy Test**	***H Pylori* Test No n (%)**	***H Pylori* Test Yes n (%)**	**Chi-Square+**	**P-Value+**	**Mantel Haenszel Chi-(p-value)**	**OR**	**95%CI**
Male	**Negative**	85(81)	23(100)	-	0.023	4.27 (0.039)	2.169*	0.316-14.896
	Positive	20(19)	0(0)					
Female	**Negative**	57(85.1)	14(93.3)	-	0.679		1**	-
	Positive	10(14.9)	1(0.7)					

+Fisher's Exact Test, *risk estimate, **reference value

3.4. Factors Associated with Asthma

An Asthma and Allergy specialist confirmed all the cases of the disease, and the factors associated with doctor-diagnosed asthma were studied using the multiple logistic regression (MLR) after adjusting for the confounding variables; sex, age, family history, and body mass index (BMI). The obtained results showed that the family history of asthma, *H pylori*, allergy test were significantly shown to have a higher predicting value for asthma (*p* values of 0.001, <0.001 and 0.01, respectively) (Tables 5). Also, the Odd-Ratio Mantel Haenszel (ORMH) of all of the three comparisons were obtained (ORMH *H pylori* test positive = 0.2, ORMH positive family history of asthma = 6.8 and ORMH allergy test positive = 3.1).

Table 5. Factors associated with doctor-diagnosed asthma using the multiple logistic regression (MLR)

Variable	Adj. OR	95% CI (OR)	X^2. Stat. (df)[a]	P. value[a]
Positive H pylori	0.2	0.5-0.1	13.3	0.001
Family history of asthma	6.8	3.4-13.8	30.5	<0.001
Positive Allergy test	3.1	1.3-7.7	6.2	0.01

a Likelihood ratio (LR) test

4. Discussion

A large number of studies demonstrated an inverse relationship between the *H. pylori* infection and asthma or atopy among children, but this relationship remains controversial due to conflicting evidences.

Our study showed that there is an inverse association between *H. pylori* sero-positivity and atopy, and that this association is more apparent in males. Moreover the *H pylori* infection at ages below nine years protect children from being asthmatic. This is in agreement with the previous studies of (Chen and Blaser, 2008; Shiotani *et al*., 2008). Furthermore the results of the multiple logistic regression showed that the main risk factors for the doctor-diagnosed asthma were *H. pylori* sero-negativity, a positive atopy test, and a positive family history of asthma. These results were consistent with other studies.

David Strachan, the founder of the historic hygiene hypothesis, was the first to postulate that the infections acquired in early childhood could prevent atopy. In the study published in 1989, he noticed that hay fever was inversely related to the number of children in the household, and to the number of older children in families. He speculated that the declining of family size associated with the improvement of cleanliness had reduced the prevalence of cross infections in families, leading to a prevalence of the atopic disease, as is the case for hay fever (Strachan, 1989). Some years later, von Mutius *et al.* (Von Mutius *et al.*, 1994) elaborated that hypothesis studying the prevalence of atopic sensitization (screened by skin prick tests) in two groups of people: children living in West Germany, and children living in East Germany, in relation to the number of siblings. He observed that atopic sensitization was three times more prevalent in West Germany than in East Germany, because of the higher standard of living.

Several case control and cross-sectional studies have investigated the relationship between the *H. pylori* infection and allergic diseases. Most of them have been conducted in industrialized countries (United States (Reibman et al., 2008), Finland (Kosunen *et al*., 2002), Great Britain (Jarvis *et al.,* 2004), Japan (Imamura *et al*.,

2010)), where atopy was found to be more common, and the decreasing trend of the *H. pylori* infection being effective. Based on these studies and others as well, the current study concludes that there is some evidence of an inverse association between atopy/allergic diseases and the *H. pylori* infection (Chen and Blaser, 2008), even though further investigations are still needed.

Furthermore, several studies demonstrated and confirmed the development of an allergic disease, or increase of IgE after the *H. pylori* eradication. A Korean study demonstrated increased levels of IgE related, non IgE related allergies as well as a subclinical rise of IgE levels in patients after the *H. pylori* eradication, compared with the *H. pylori*-positive patients without eradication and *H. pylori* negative controls (Lee *et al*., 2015).

In addition, some studies stated that the inverse relation between *H. pylori* and allergy is conditional; for instance one study proved that the significant negative association was demonstrated in men, but not in women, underlining a difference in the negative association in relation to sex, which suggests a different immune response to *H. pylori* in women more than in men (Shiotani *et al*., 2008). Other studies showed that the negative association was stronger in child asthma than in adult asthma, as the adult asthma may be multifactorial and child asthma is mostly attributed to atopy (Chen and Blaser, 2008); for instance, Blaser considered that the inverse association observed with early life asthma (not with long-standing asthma seen in adults) supported the role of *H. pylori,* since the effect of *H. pylori* might be less important in adult-onset asthma due to the much more heterogeneous nature of adult asthma. However, the confounding factors that could influence the association are not fully ruled out.

Potential mechanisms by which *H. pylori* could alter the presentation of asthma include immune modifications, or an effect on the gastroesophageal reflux disease (GERD). The exogenous infection and microbial substances including the *H. pylori* infection may elicit a Th1-mediated immune response, which suppresses Th2 responses (Amedei *et al*., 2006; Codolo *et al*., 2008). The lack of adequate stimulation of the Th1 might result in an overactive Th2, which in turn leads to allergy (D'Elios and Bernard, 2010). Moreover, the acquisition of *H. pylori* may be of importance in the induction of regulatory T cells, which could effectively reduce the possibility of allergy (Moyat and Velin, 2014). The immune-modulatory properties that allow the bacteria to persist for decades in infected individuals in the face of a vigorous, yet ultimately non-protective, innate, and adaptive immune response may at the same time confer protection against allergies, asthma, and inflammatory bowel diseases. Regulatory T-cells mediating peripheral immune tolerance have emerged as key cellular players in facilitating persistent infection as well as protection from allergies in humans as appears from the observational studies and in mice as the experimental studies have shown (Arnold and Anne Müller, 2012; Slomiany and Slomiany, 2014). Associations between GERD and asthma are also well-established (Harding, 2005). Longitudinal studies show that asthma is a risk factor for the development of GERD, and that GERD can trigger asthma. *H. pylori*; in particular CagA+ strains, are inversely associated with GERD (Haruma *et al*., 2000; Peek Jr and Blaser, 2002; Blaser and Atherton, 2004). Although we did not specifically investigate GERD in this study, there is a possibility that the inverse association between *H. pylori* and asthma reflects protection from GERD.

H. pylori may be asymptomatic; however, children infected with *H. pylori* can manifest gastrointestinal diseases. Although this study showed a significant association between *H. pylori* related gastrointestinal symptoms (except for poor growth; data not shown) and *H. pylori* seropositivity, controversy persists regarding testing (and treating) for the *H. pylori* infection in children with recurrent dyspepsia, chronic idiopathic thrombocytopenia, regurgitation, gastric ulcer and/or anemia. Because of the not conclusive evidences of the role of *H. pylori* in childhood gastrointestinal disease, several studies have demonstrated that *H. p ylori* infection is not associated with specific symptomatology in children (Pacifico *et al*., 2010). Therefore, the identification of children with *H. pylori*-associated gastritis on the basis of clinical presentation alone is not possible. Based on the best available evidence, testing for (and treating) *H. pylori* infections should be performed in children with endoscopically proven duodenal ulcer. Evidence from studies on adults supports the recommendation that testing for *H. pylori* should also be performed in children with a documented gastric ulcer. Endoscopy and biopsy are also recommended for children with persistent symptoms (Patel *et al.*, 1994)

This study has several limitations that should be mentioned, for instance the cross-sectional study design. Because this study only checked the history of asthma, it could not exclude the possibility of the development of asthma before the *H. pylori* infection. However, as *H. pylori* acquisition is known to occur early in life, the development of asthma was presumed to be the later event in the present study. Most of the subjects were not highly-educated living in a rural area. This might have caused a selection bias. Recall bias may have occurred, as our data on the history of allergic diseases (hay fever, eczema and food allergy) are depended on self-reports. Furthermore, the assessment of patients for the *H. pylori* infection using the biopsy method, which has a higher sensitivity and specificity than serology tests, is needed to prove the results. However, after adjusting for those factors, the *H. pylori* infection was inversely related to asthma and atopy. Performing more studies with larger sample sizes is necessary to confirm these results.

5. Conclusion

In conclusion, our data suggest that *H. pylori* is inversely associated with atopy and asthma. It also shows that this inverse association was more apparent in males and in the less-than-nine age group. Moreover, the *H. pylori* seronegativity, positive family history of asthma, and positive atopy test are all triggering factors for asthma (after removing the confounding factors). Obviously, a precise recognition of the correlation between the *H. pylori* infection and asthma in young children could play an important role in the recognition of the physiopathology of not only asthma, but of other allergic diseases as well. This would offer potentially helpful new treatments for allergic diseases. Therefore, we recommend a careful consideration

of whether to eradicate *H. pylori* in young patients with asthma risk factors, as *H. pylori* may play a role in reducing the risk for asthma. Further prospective studies are warranted to clarify the underlying mechanisms.

References

Alsaimary I E, Mezal T J, Jassim H A, Alwajeeh Y A, Talib R A, Abdul-Samad H Q, Hussain D S and Majeed H A. 2014. Seroprevalence of *Helicobacter pylori* among healthy medical students in Al-Basrah Province. *Donnish J Microbiol Biotechnol Res.,* **1(2)**: 012-017.

Amedei A A, Cappon G, Codolo A, Cabrelle A, Polenghi M, Benagiano E, Tasca A,Azzurri M M, D'Elios and G Del Prete. 2006. The neutrophil-activating protein of *Helicobacter pylori* promotes Th1 immune responses. *J Clin Invest*., **116(4)**: 1092.

Arnold I C and Anne Müller I H. 2012. The immunomodulatory properties of *Helicobacter pylori* confer protection against allergic and chronic inflammatory disorders. *Res Advances in the Study of Campylobacter, Helicobacter & Related Organisms*., 135.

Asher M U, Keil H, Anderson R, Beasley J, Crane F, Martinez E, Mitchell N, Pearce B, Sibbald and A Stewart. 1995. International Study of Asthma and Allergies in Childhood (ISAAC): rationale and methods. *Eur Respir J*., **8**(3): 483-491.

Baccioglu A F, Kalpaklioglu S, Guliter and F Yakaryilmaz. 2008. Helicobacterpylori in allergic inflammation–Fact or fiction? *Allergologia et Immunopathol..,* **36**(2): 85-89.

Blaser M J and Atherton J C. 2004. *Helicobacter pylori* persistence: biology and disease. *J Clin Invest.,* **113**(3): 321.

Blaser M J, Chen Y and Reibman J. 2008. Does *Helicobacter pylori* protect against asthma and allergy?. *Gut*., **57(5)**: 561-567.

Chen Y and Blaser M J. 2008. *Helicobacter pylori* colonization is inversely associated with childhood asthma. *J infect Dis.,* **198**(4): 553-560.

Codolo G P, Mazzi A, Amedei G, Del Prete G, Berton M, D'Elios M and De Bernard M . 2008.The neutrophil-activating protein of *Helicobacter pylori* down-modulates Th2 inflammation in ovalbumin-induced allergic asthma. *Cellular Microbiol.,* **10**(11): 2355-2363.

D'Elios M M and Bernard M d. 2010. To treat or not to treat Helicobacter Pylori to benefit asthma patients. *Expert Review Respiratory Med.,* **4**(2): 147-150.

Daugule I J, Zavoronkova and Santare D. 2015. *Helicobacter pylori* and allergy: Update of research. *World J Methodol.,* **5(4)**: 203.

Dooley, C P, Cohen H, Fitzgibbons P L, Bauer M, Appleman M D, Perez-Perez G I and Blaser M J. 1989. Prevalence of *Helicobacter pylor*i infection and histologic gastritis in asymptomatic persons. *N Engl J Med .,* **321**(23): 1562-1566.

Harding S M. 2005. Gastroesophageal reflux: a potential asthma trigger. *Immunol Allergy Clin North Amer.,* **25**(1): 131-148.

Haruma K, Hamada H, Mihara M, Kamada T, Yoshihara M, Sumii K, Kajiyama G and Kawanishi M. 2000. Negative association between *Helicobacter pylori* infection and reflux esophagitis in older patients: Case-Control study in Japan. *Helicobacter*., **5**(1): 24-29.

Herbrink P and Van Doorn L. 2000. Serological methods for diagnosis of *Helicobacter pylori* infection and monitoring of eradication therapy. *Europ J Clin Microbiol & Infect Dis.,* **19**(3): 164-173.

Imamura S, Sugimoto M, Kanemasa K, Sumida Y, Okanoue T, Yoshikawa T and Yamaoka Y. 2010. Inverse association between *Helicobacter pylori* infection and allergic rhinitis in young Japanese. *J Gastroenterol Hepatol.,* **25**(7): 1244-1249.

Jarvis D, Luczynska C, Chinn S and Burney P. 2004. The association of hepatitis A and *Helicobacter pylori* with sensitization to common allergens, asthma and hay fever in a population of young British adults. *Allergy*, **59**(10): 1063-1067.

Jun Z J, Lei Y, Shimizu Y, Dobashi K and Mori M. 2005. *Helicobacter pylori* seroprevalence in patients with mild asthma. *Tohoku J Exp Med.,* **207**(4): 287-291.

Kosunen T, Höök-Nikanne J, Salomaa A, Sarna S, Aromaa A and Haahtela T. 2002. Increase of allergen-specific immunoglobulin E antibodies from 1973 to 1994 in a Finnish population and a possible relationship to *Helicobacter pylori* infections. *Clin Exp Allergy*., **32**(3): 373-378.

Kuipers E J, Pérez-Pérez G I, Meuwissen S G and Blaser M J. 1995. *Helicobacter pylori* and atrophic gastritis: importance of the cagA status. *J National Cancer Institute*., **87**(23): 1777-1780.

Lee S P, Lee S Y, Kim J H, Sung I K, Park H S, Shim C S and Moon H W. 2015. Correlation between *Helicobacter pylori* infection, IgE hypersensitivity, and allergic disease in Korean adults. *Helicobacter*., **20**(1): 49-55.

Mallol J, Crane J, von Mutius E, Odhiambo J, Keil U, Stewart A and I P T S Group. 2013. The International Study of Asthma and Allergies in Childhood (ISAAC) phase three: a global synthesis. *Allergologia et Immunopathol.,* **41**(2): 73-85.

Matsushima K and Nagai S. 2012. Unraveling the mystery of the hygiene hypothesis through *Helicobacter pylori* infection. *J Clin Invest.,* **122**(3): 801.

Miftahussurur M, Nusi I A, Graham D Y and Yamaoka Y. 2017. *Helicobacter*, Hygiene, Atopy, and Asthma. *Frontiers in Microbiol.,* **8**: 1034.

Moyat M. and Velin D. 2014. Immune responses to *Helicobacter pylori* infection. *World J Gastroenterol* **., 20**(19): 5583-5593.

Pacifico L, Anania C, Osborn J F, Ferraro F and Chiesa C. 2010. Consequences of *Helicobacter pylori* infection in children. *World J Ggastroenterol*., **16**(41): 5181.

Patel P, Mendall M, Khulusi S, Northfield T and Strachan D. 1994. *Helicobacter pylori* infection in childhood: risk factors and effect on growth. *BMJ*, **309**(6962): 1119-1123.

Peek Jr R M, and Blaser M J. 2002. *Helicobacter pylori* and gastrointestinal tract adenocarcinomas. *Nature Reviews Cancer*, **2**(1): 28.

Raj S M, Choo K E, Noorizan A M, Lee Y Y and Graham D Y. 2009. Evidence against *Helicobacter pylori* being related to childhood asthma. *J Infect Dis.,* **199**(6): 914-915.

Reibman J, Marmor M, Filner J, Fernandez-Beros M E, Rogers L, Perez-Perez G I and Blaser M J. 2008. Asthma is inversely associated with *Helicobacter pylori* status in an urban population. *PloS one*., **3**(12): e4060.

Rigoli L, Briuglia S, Caimmi S, Ferrau V, Gallizzi R, Leonardi S, La Rosa M and Salpietro C. 2011. Gene-environment interaction in childhood asthma. *Inter J Immunopathol Pharmacol.,* **24**(4 suppl): 41-47.

Shiotani A, Miyanishi T, Kamada T and Haruma K. 2008. *Helicobacter pylori* infection and allergic diseases: epidemiological study in Japanese university students. *J Gastroenterol Hepatol.,* **23**(7pt2).: e29-33

Slomiany B and Slomiany A. 2014. Role of ghrelin-induced phosphatidylinositol 3-kinase activation in modulation of gastric mucosal inflammatory responses to *Helicobacter pylori. Inflammopharmacol*., **22**(3): 169-177.

Strachan D P. 1989. Hay fever, hygiene, and household size. *BMJ* **., 299** (6710): 1259.

Suerbaum S and Michetti P. 2002. *Helicobacter pylori* infection. *N Engl J Med* ., **347**(15): 1175-1186.

Von Mutius E, Martinez F D, Fritzsch C, Nicolai T, Reitmeir P and Thiemann H H. 1994. Skin test reactivity and number of siblings. *BMJ*., **308**(6930): 692-695.

JJBS

Jordan Journal of Biological Sciences

Volume 11, Number 2,June 2018
ISSN 1995-6673
Pages 129 - 139

The Corrective Efficacy of the *n*-Hexane Fraction of the Hydro-Methanol Extract of the *Swietenia mahagoni* Seeds on Testicular Dysfunctions in Streptozotocin-Induced Diabetic Male Rats

Tushar Kanti Bera[1] and Debidas Ghosh[2,*]

[1]*Department of Physiology, Universal College of Medical Sciences,Lumbini Zone, Bhairahawa, Nepal;*

[2]*Department of Bio-Medical Laboratory Science and Management, Vidyasagar University, Midnapore-721 102,West Bengal, India*

Received May 23, 2017; Revised October 12, 2017; Accepted October 22, 2017

Abstract

Diabetes mellitus (DM) is one of the most common chronic metabolic diseases worldwide affecting male reproductive dysfunctions (RD). Male diabetics are more prone to having varieties of sexual disorders such as impotence, retrograde ejaculation, sexual drive, decreased libido, delayed sexual maturation, and erectile dysfunction (ED). The present study is designed to investigate the diabetes-induced testicular dysfunction and its treatment by the n-hexane fraction of the hydro-methanolic (2:3) seed extract of *Swietenia mahagoni* in Wistar male albino rats. Diabetes was induced by the intramuscular injection of streptozotocin (STZ) at a dose of 4 mg/mL of citrate buffer/100 gm body weight. Diabetes-induced testicular oxidative stress injuries were assessed by the determination of antioxidant enzyme-activities including catalase (CAT), superoxide dismutase (SOD), and glutathione-s-transferase (GST), along with thiobarbituric acid-reactive substances (TBARS), and conjugated dienes (CD) levels. The numbers of the different generations of germ cells [i.e. spermatogonia-A (SgA), preleptotine spermatocytes (pLSc), mid-pachytene spermatocyte (mPSc), step 7 spermatids (7Sd) and 19 spermatids (19Sd)], at stage VII of spermatogenic cycle were assessed. Testicular CAT, SOD and GST activities were decreased along with elevation in the levels of TBARS and CD in diabetic animals. Numbers of different generations of germ cells i.e. SgA, pLSc, mPSc, 7Sd and 19Sd, at stage VII of the spermatogenic cycle were decreased in diabetic animals. Significant improvement in the levels of blood glucose, serum insulin, testosterone, and testicular oxidative stress parameters were noted towards control after the treatment of diabetic rats with the fraction at a dose of 10 mg/100 gm body weight/day for twenty-eight days. The above-mentioned different generations of germ cell numbers along with the diameter and number of pancreatic islets were recovered towards control after the treatment with the fraction. The results support the validity of this n-hexane fraction for the management of testicular disorders noted in diabetic rats.

Keywords: *Swietenia mahagoni* (L.) Jacq., Streptozotocin, Antioxidant enzymes, Pancreas, Testis

1. Introduction

Diabetes mellitus (DM) represents one of the greatest threats to modern global health. The estimated number of adults with diabetes in 2007 was 246 million; of these, 80% live in developing countries, with the largest numbers being from the Indian subcontinent and China. India has forty-one million diabetics, and this number it is expected to increase to seventy million by 2025 (Sicree *et al.,* 2006). According to the World Health Organization (WHO) estimates, the urban population in developing regions will increase from 1.9 billion in 2000 to 3.9 billion in 2030. It is estimated that, by 2030, nearly 46% of India's population will be living in urban areas (WHO, 2002). The Diabetes' incidence is rising rapidly. It is a heterogeneous group of diseases, known as a syndrome, and has become one of the greatest threats to modern global health and the third leading cause of death (after heart disease and cancer) in many countries (Can *et al.,* 2004).

DM may affect the male reproductive function at multiple levels as a result of its effects on the endocrine control of spermatogenesis, or by impairing penile erection and ejaculation (Soudamani *et al.,* 2005). There are a number of reports in the related literature examining the effects of diabetes on the endocrine control of spermatogenesis (Baccetti *et al.,* 2002). Diabetes is, however, a well-recognized cause of male sexual dysfunction, which in itself may contribute to subfertility. Data from animal models strongly suggest that DM impairs male fertility. Furthermore, numerous studies have demonstrated a marked reduction in fecundity when male

* Corresponding author. e-mail: debidas_ghosh@yahoo.co.in.

animals are diabetic, as well as an impairment of sperm quality (Gnoth *et al.*, 2005). If similar effects exist in the context of human male reproduction, the rising rates of diabetes may well pose a significant problem to human fertility. Despite this, the potential impact of the increase in diabetes in young men and its effect on their reproductive health have received comparatively little attention to date.

However, there are several reports about the dysfunctions of the male reproductive activity in diabetic conditions, but the data are confusing, and the exact role that insulin plays in the regulation of male reproductive function is still unclear. Insulin is known to influence the hypothalamic-pituitary axis (Bucholtz *et al*., 2000), which can alter serum hormone levels significant in spermatogenesis. It is also known that insulin may be able to interact with receptors on the Leydig cells directly to mediate the process of steroidogenesis; thus, we cannot exclude the possibility that plasma insulin also directly interacts with Leydig cells, in addition to the indirect signaling through the pituitary. Leydig cells contain insulin receptors (Brüning *et al*., 2000), and the administration of insulin to Leydig primary cultures causes an increase in testosterone formation (Pakarainen *et al.*, 2005). In the practices of traditional medicine, medicinal plants are used in many countries to control diabetes mellitus. The National Centre for Complementary and Alternative Medicine was established in 1998 by the United States Government for the development of herbal medicine as an important subject of study (Sing *et al.* 2005; Mallick *et al.*, 2010). Recently, there is a greater global interest in non-synthetic, natural drugs derived from plants and herbal sources due to their better tolerance and minimum potential adverse drug reactions (Pari and Umamaheswari, 2000). Plants play a significant role in human health, and have been considered valuable sources of natural products for maintaining a good health for many years. The WHO suggested that medicinal plants would be the best source from which to develop a variety of medications (WHO, 2001).

The plant, *Swietenia mahagoni* (L.) Jacq. from the (Family- Meliaceae), is a lofty, evergreen large tree, native to tropical America, Mexico, and South America, as well as India (Kirk, 2009). The seed extracts of *S. mahagoni* are widely used in Indonesia as folk medicine to cure diabetes (Li *et al.*, 2005). In an earlier study, the authors have reported the antidiabetic activity of n-hexane fraction of the hydro-methanol (2:3) extract of *S. mahagoni* seeds on an animal model (Bera *et al.*, 2015). Thus, this study has been undertaken to investigate the diabetes-related testicular disorders, and to evaluate the remedial effects of n-hexane of the hydro-methanol (2:3) extract of the *S. mahagoni* seeds on testicular dysfunctions in diabetic conditions.

2. Materials and Methods

2.1. Plant Materials

The seeds of *Swietenia mahagoni* (L.) Jacq. of the family- Meliaceae were collected from Medinipur in the District of Paschim Medinipur in West Bengal, India during December 2016. The materials were taxonomically identified by Prof. R. K. Bhakat of the Department of Botany and Forestry at Vidyasagar University in Medinipur. The voucher specimen was deposited in the Department of Botany at Vidyasagar University (Ref. No. *S. mahagoni* (L.) Jacq. / VU / 01 / 09).

2.2. Chemicals

Streptozotocin (STZ) was purchased from Sigma–Aldrich Diagnostic Ltd. USA. The insulin- enzyme linked immunosorbent assay (ELISA) kit was purchased from Boehringer Mannheim Diagnostic, Mannheim (Germany). The testosterone kit was purchased from IBL–Germany. The biochemical kits were purchased from Span Diagnostic Ltd. Surat, India. The blood- glucose levels were measured using a one touch electronic glucometer of Ascensia Entrust, Bayer Diagnostics Ltd., Borada, India.

2.3. The Selection of Animal and Animal Care

The current study was conducted on matured Wistar strain male albino rats of three months of age weighing approximately 150 ± 10 g. The rats were acclimated for a period of fifteen days in this study's laboratory conditions prior to the experiment. They were housed in Tarson cages (Tarson Products Pvt. Ltd., Kolkata, India) at an ambient temperature of 25 ± 2 ^{0}C with a twelve-hour light: twelve-hour dark cycle. The rats were allowed free access to standard food and water *ad libitum.* Normoglycemic animals selected for this experiment had a fasting blood glucose level of 75±5 mg/dL.

2.4. Preparation of the n-Hexane Fraction of the Hydro-Methanol (2:3) Extract from the Seeds of Swietenia mahagoni (L.) Jacq.

Fresh seeds of *S. mahagoni* were dried in an incubator for two days at 40 ^{0}C, crushed separately in an electric grinder, and then pulverized. Out of this powder, 500 g were suspended in 400 mL of water and 600 mL methanol (2:3) and were kept in an incubator at 37 ^{0}C for thirty-six hours. The slurry was stirred intermittently for two hours and was left overnight. About 96.8 g of the light brown colored lyophilized extract was collected. In a one-litre separate flask, the extract (96.8 g) was dissolved with 500 mL of a hydro-methanolic (2:3) solution, and the solvent fractionation was carried out using solvents (*n*-hexane, chloroform, ethyl acetate and *n*-butanol) with increasing polarity. Finally, from the 96.8 g- lyophilized extract of *S. mahagoni*, an amount of 27.6 g of *n*-hexane fraction was obtained.

2.5. Induction of Diabetes Mellitus

The Streptozotocin-induced diabetes was done by the standard method as mentioned earlier (Panda *et al.*, 2009). In brief, twenty-five rats fasting for twenty-four hours were subjected to a single intramuscular injection of streptozotocin (STZ) at a dose of 4 mg/ 0.1 mL of citrate buffer (pH 4.5) / 100 g body weight / rat. On the seventh day of the STZ injection, fasting blood sugar (FBG) levels were measured, and the eighteen diabetic rats were placed in group II to group IV having six rats in each group. The remaining seven rats having FBG less than 250 mg/ dL were not selected for this experiment. Six other normoglycemic rats were subjected to a single injection of citrate buffer at a dose of 0.1 mL/ 100 g body weight/ rat at

the time of the STZ injection in other rats, and these are kept under the vehicle control group.

2.6. Animal Treatment

Twenty-four rats were divided into four groups equally as follows; the duration of the experiment was thirty-five days.

Vehicle control: Normoglycemic healthy rats of this group were treated with 0.5 mL of 3.0% DMSO solution / 100 gm body weight / day for twenty-eight days at the time of the n-hexane fraction treatment of the diabetic rats.

Vehicle diabetic: Diabetic rats of this group were treated with 0.5 mL of 3.0% DMSO solution/ 100 gm body weight/ day for twenty-eight days at the time of the *n*-hexane fraction treatment of the diabetic rats.

Diabetic + *n*-hexane fraction: Diabetic rats of this group were treated with n-hexane fraction of the hydro-methanol (2:3) extract of the *S. mahagoni* seeds at a dose of 10 mg / 0.5 mL of 3.0% DMSO solution/ 100 gm body weight / day for twenty-eight days.

Diabetic + metformin: Diabetic animals of this group were treated with metformin (standard antidiabetic drug) at a dose 1 mg / 0.5 mL of 3.0 % DMSO solution / 100 gm body weight / day for twenty-eight days.

The fraction was administered orally using a feeding cannula daily for twenty-eight days starting from the eighth day of the STZ injection considering the first day of the fraction/ metformin treatment. From the first day of the fraction / metformin treatment of the diabetic rats, a fasting blood glucose level was measured every seventh day using a glucometer (Bera *et al*., 2010). On the twenty-ninth day of the experiment (the thirty-sixth day following the day of the STZ injection), all the animals were sacrificed by decapitation after recording the final body weight. Blood was collected from the dorsal aorta using a syringe. Serum was separated from part of the collected blood by centrifugation at 3000 g for five minutes to evaluate the levels of the serum insulin, and testosterone, in addition to the activities of serum glutamic pyruvic transaminase (SGPT), and serum glutamic oxaloacetic transaminase (SGOT. Epididymis and testes were dissected out, and one testis was stored at –20 ^{0}C for the biochemical analysis, and other testes were used for the histological study. Cauda epididymis of each animal was stored at 37^{o}C in buffer, and was used for epididymal sperm count, motility and viability.

2.7. Measurement of the Fasting Blood Glucose (FBG) Level

At the time of grouping the animals, the FBG level was measured. On every seventh day of treatment, FBG was further recorded from all the animals in all groups. Blood was collected from the tip of the tail vein, or by an orbital puncture alternatively, and the FBG levels were measured by the one-touch glucometer (Bera *et al*., 2010).

2.8. Serum Insulin Level

Serum insulin was measured by an enzyme-linked immunosorbent assay (ELISA) using the kit (Boehringer Mannheim Diagnostic, Mannheim, Germany) (Briner *et al*., 1988). The insulin level in the serum was expressed in µIU/mL.

2.9. Serum Testosterone Level

Serum levels of testosterone were measured using the testosterone kit from IBL- Germany according to the standard protocols supplied by that company (Srivastava, 2001). In this solid phase-conjugated assay, an alkaline phosphatase conjugated hormone was used. There is no inter-assay variation as all the samples were assayed at a time.

2.10. Sperm Viability

The sperm viability was performed by the eosin nigrosin staining (WHO, 1999). One drop of semen was mixed with two drops of 1% eosin Y. After thirty seconds, three drops of 10% nigrosin were added and mixed well. A smear was made by placing a drop of the mixture on a clean glass slide, and was allowed to air-dry. Pink-stained dead sperms were differentiated from unstained live sperms, and their numbers were recorded.

2.11. Estimation of Testicular Δ5, 3β- Hydroxysteroid Dehydrogenase (Δ5, 3β- HSD) and 17 β- Hydroxysteroid Dehydrogenase (17 β- HSD) Activities

The testicular Δ^5, 3β- HSD activity was measured spectrophotometrically (Talalay, 1962). One testis from each animal was homogenized carefully at 4^{o}C in 20% spectroscopic grade glycerol containing 5 mM potassium phosphate and 1 mM EDTA, at a tissue concentration of 100 mg/mL homogenizing mixture. This mixture was centrifuged at 10,000 X g for thirty minutes at 4^o C. The supernatant (1ml) was mixed with 100 µM sodium pyrophosphate buffer (pH 8.9), 40 µL ethanol, containing 30 µg dehydro-epiandrosterone, and 960 µL of 25 mg% BSA, bringing the incubation mixture to a total of 3 mL. One unit of the enzyme activity was the amount causing a change in absorbance of 0.001 / minute at 340 nm.

For the measurement of the testicular 17 β- HSD activity, another 1mL supernatant from the same homogenizing mixture was added to 440 µM sodium pyrophosphate buffer (pH 10.2), 40 µL ethanol containing 0.3 µM testosterone, and 960 µL of 25 mg% BSA, bringing the incubation mixture to a total of 3 mL. The enzyme activity was measured according to the method of Jarabak *et al.* (1962). One unit of the enzyme activity was equivalent to a change in absorbency of 0.001 / min at 340 nm.

2.12. Biochemical Assay of Testicular Catalase (CAT) Activity

The activity of testicular catalase was measured biochemically (Beers and Sizer, 1952). 0.5 mL of 0.00035 M H_2O_2 and 2.5 mL of distilled water were mixed in a spectrophotometric cuvette, and the reading of absorbance was noted at 240 nm. The supernatants of the testicular samples were added at a volume of 40 µL, and the subsequent six readings were noted at 30 sec interval.

2.13. Assessment of Testicular Superoxide Dismutase (SOD) Activity

The testis was homogenized in a chilled 100 mM/L tris HCl buffer containing 0.16 M/L potassium chloride (pH 7.4) to give a tissue concentration of 10% (weight/volume), and was centrifuged at 10,000 rpm for twenty minutes at 4^{0}C. The SOD activity of the sample was estimated by measuring the percentage inhibition of

the pyrogallol auto oxidation by SOD according to the standard method (Marklund and Marklund, 1974).

2.14. Determination of Testicular Glutathione-s-Transferase (GST) Activity

The activity of GST in the testis tissue was measured spectrophotometrically using 1-chloro-2, 4-dinitrobenzene (CDNB) as a substrate (Hobig *et al*., 1974). The assay mixture of 3 mL contained 0.1 mL of 1 mM CDNB in ethanol, 0.1mM of 1 mL GSH, 2.7 mL of 100 mM potassium phosphate buffer (pH -6.5) and 0.1 mL of the supernatant of the tissue homogenate. The formation of the product of CDNB, S-2, 4-dinitrophenylglutathione, was monitored by measuring the net increase in absorbance at 340 nm against the blank.

2.15. Estimation of Testicular Lipid Peroxidation from the Concentration of Thiobarbituric Acid Reactive Substance (TBARS) and Conjugated Dienes (CD)

The testis was homogenized separately with a tissue concentration of 50 mg / mL in 0.1 M of ice-cold phosphate buffer (pH-7.4), and the homogenates were centrifuged at 10,000 g at 4^0C for five minutes individually. Each supernatant was used for the estimation of TBARS and CD (Okhawa *et al.,* 1979; Slater, 1984). For the measurement of TBARS, the homogenate mixture of 0.5 mL was mixed with 0.5 mL of normal saline (0.9 g% NaCl) and 2 mL of TBA-TCA mixture (0.392 g thiobarbituric acid in 75 mL of 0.25 N HCl with 15 g trichloroacetic acid (TCA). The volume of the mixture was made up to 100 mL by 95% ethanol) and boiled at 100 ^{0}C for 10 min. This mixture was then cooled at room temperature and was centrifuged at 4000 g for ten minutes. The whole supernatant was taken in a spectrophotometer cuvette, the reading was at 535 nm (Okhawa *et al*., 1979).

The quantification of the CD was performed by a standard method (Slater, 1984). The lipids were extracted with chloroform-methanol (2:1) followed by centrifugation at 1000 g for five minutes. The chloroform layer was evaporated to dryness under a stream of nitrogen. The lipid residue was dissolved in 1.5 mL of cyclohexane and the absorbance was noted at 233 nm to measure the amount of the hydro-peroxide formed.

2.16. Biochemical Assay of Serum Glutamate Oxaloacetate Transaminase (SGOT) and Serum Glutamate Pyruvate Transaminase (SGPT)

The activities of GOT and GPT in serum were measured by specific kits supplied by Span Diagnostic Ltd., Surat, India. The activities of these enzymes were expressed as IU/L of serum (Henry *et al*., 1960).

2.17. Histopathology of Testis and Pancreas

The tissues were subjected to paraffin embedding followed by section cutting (5 µm thick) with Leica microtome (Leica Biosystem). Deparaffinized sections were stained with hematoxylin and eosin. Histological examinations were carried out on stained sections with a computer-aided microphotography system using Avar Cap (Avatar Capture, Version-2.5; Aver Media Technologies Inc.), and Dewinter Caliper Pro 3.0 software (Dewinter Optical Inc.). The quantification of different generations of germ cells at stage VII was performed according to the method of Leblond and Clermont (1952). The cells present in this stage are spermatogonia-A (SgA), preleptotine spermatocytes (pLSc), mid-pachytene spermatocyte (mPSc), step seven spermatids (7Sd) and nineteen spermatids (19Sd). The different nuclei of the germ cells (except step ninteen spermatids, which cannot be precisely counted) were counted at twenty round tubular cross-sections in each rat. All the nuclear count of germ cells was corrected for differences in nuclear diameter by the formulae of Abercrombie (1947). True count= (Crude count × section thickness) / (section thickness + diameter of germ cell), and tubular shrinkage by the Sertoli cell correction factor (Clermont and Morgentaler, 1955).

The diameters of the pancreatic islets were measured by the Avar Cap (Avatar Capture; (Version-2.5); Aver Media Technologies Inc.) and Dewinter Caliper Pro 3.0 software (Dewinter Optical Inc.). Islet cells were counted per islet under 1000X magnifications. Islet count per specific parts of the pancreas was performed by a total scanning of the section under a microscope (Mallick *et al*., 2009).

2.18. Phytochemical Analysis of n-hexane Fraction of Hydro-methanol (2:3) Extract of the Seeds of Swietenia mahagoni (L.) Jacq.

2.18.1. Test for Alkaloids

The 0.25 g *n*-hexane fraction was defatted with 5 % ethyl ether for fifteen minutes. The defatted sample was extracted for twenty minutes with 5 mL of aqueous HCl using a boiling water bath. The resulting mixture was centrifuged for ten minutes at 3000 rpm (Trease and Evans, 2002).

2.18.2. Test for Flavonoids

A portion of the crude powder was heated with 10 mL of ethyl acetate over a steam bath for three minutes. The mixture was filtered and 4 ml of the filtrate was shaken with 1 mL of dilute ammonia solution, and a yellow coloration was observed (Edeoga *et al.,* 2005).

2.18.3. Test for Tannins

A crude powder of 0.25 g in amount was stirred with 10 mL of distilled water. This was filtered and the ferric chloride reagent was added to the filtrate. A blue-black precipitate was taken as evidence for the presence of tannin (Kolawole *et al*., 2006).

2.18.4. Test for Terpenoids

0.25 g of crude powder was dissolved in 5 mL of methanol. Two mL of the extract was treated with 1 mL of 2, 4-dinitrophenyl hydrazine dissolved in 100 mL of 2M HCl. A yellow-orange coloration was observed as an indication of terpenoids (Kolawole *et al.,* 2006).

2.18.5. Test for Steroids

0.25 g of crude powder was dissolved in 5 mL of methanol. One mL of the extract was treated with 0.5 mL of acetic acid anhydride, and was cooled in ice. This was mixed with 0.5 mL of chloroform, and 1 mL of concentrated sulphuric acid was then added carefully using a pipette (Kolawole *et al.,* 2006).

2.19. Statistical Analysis

The analysis of variance followed by multiple comparisons using the two-tailed Student's T-Test for the

statistical analysis of the collected data by Origin 6.1 software (Origin Lab Corporation) (Sokal and Rohle, 1997). All of the values were indicated in the tables and figures as mean ± standard deviation (mean ± SD). Differences were considered significant at the level of $p < 0.05$.

3. Results

3.1. Body Weight and Organo-Somatic Indices

Body weight and organo-somatic indices were decreased significantly ($p<0.001$) in STZ-induced vehicle diabetic rats in comparison to the vehicle control group. The treatment of the diabetic animals with the n-hexane fraction (10 mg / 100 gm body weight / day for twenty-eight days) resulted in significant ($p<0.05$) recovery in the level of these parameters towards the control. The metformin treatment of diabetic animals resulted in resettlement of the above-mentioned parameters to the control level with the exception of the epididymis-somatic index (Table 1).

Table 1. Effect of *n*-hexane fraction of hydro-methanol (2:3) extract of *S. mahagoni* seeds on body weight and organo-somatic indices in streptozotocin-induced diabetic male albino rats.

Groups	Body weight (gm)		Testiculo-somatic index (gm%)	Epididymis-somatic index (gm%)	Seminal vesiculo-somatic index (gm%)
	Initial	Final			
Vehicle control	153.62± 4.51	161.03 ± 4.54	2.63 ± 0.17	0.65 ± 0.03	0.42 ± 0.03
Vehicle diabetic	154.21± 5.30	140.52± 3.76*	1.24 ± 0.14*	0.40 ± 0.04 *	0.20 ± 0.04 *
Diabetic +n-hexane fraction	151.94± 4.42	155.01± 4.12**	2.12 ± 0.12**	0.58 ± 0.02 **	0.36 ± 0.02 **
Diabetic + metformin	152.75± 4.36	160.22± 4.23	2.58 ± 0.16	0.59 ± 0.03**	0.41 ± 0.02

All values are expressed as mean ± SD, n = 6. Analysis of variance followed by multiple comparisons using two-tailed Student- t test, where *indicates $P< 0.001$ and **indicates $P <0.05$ compared with vehicle control group.

3.2. Fasting Blood Glucose (FBG) Level

Streptozotocin-induced diabetic animals resulted in a significant ($p<0.001$) elevation in the fasting blood glucose (FBG) level in comparison to the vehicle control animals. The level of this parameter was significantly ($p<0.05$) recovered towards the control level after the treatment of the STZ-induced diabetic animals by the n-hexane fraction. Treatment of the diabetic animals with metformin (1 mg / 100 gm body weight / day for twenty-eight days) resulted in resettlement in the level of FBG compared to the control level on the thirty-fifth day. Moreover, there was no significant difference in the level of FBG between the vehicle control and the metformin- treated diabetic groups on the thirty-fifth day (Table 2).

Table 2. Remedial effect of *n*-hexane fraction of hydro-methanol (2:3) extract of *S. mahagoni* seeds on fasting blood glucose level in streptozotocin-induced diabetic male albino rats.

Groups	Fasting blood glucose (FBG) level (mg / dL)					
	1st day	7th day	14th day	21st day	28th day	35th day
Vehicle control	75.3± 3.3	74.5± 3.4	76.6± 3.2	73.4± 3.1	75.3± 3.3	74.6± 3.2
Vehicle diabetic	73.6± 3.6	302.7± 4.3*	321.4± 4.7*	338.5± 4.8*	356.5± 4.9*	371.8± 4.8*
Diabetic +n-hexane fraction	74.1± 4.1	296.3± 3.8*	228.5± 3.8*	176.7± 3.7*	118.9±3.5*	95.2± 3.6**
Diabetic + metformin	76.4± 3.5	298.5± 4.1*	201.7± 3.5*	138.3± 3.6*	94.6±3.4**	76.4± 3.1

All values are expressed as mean ± SD, n = 6. Analysis of variance followed by multiple comparisons using the two-tailed Student-t test, where *indicates $P< 0.001$ and **indicates $P <0.05$ compared with vehicle control group.

3.3. Levels of Serum Insulin and Testosterone

The levels of serum insulin and testosterone were decreased significantly in the vehicle diabetic animals in comparison to the vehicle control animals. The oral administration of n-hexane fraction at the above-mentioned dose to the diabetic animals resulted in substantial correction of the levels of these parameters towards the control level. No significant variations were noted in the levels of serum insulin and testosterone between the n-hexane fraction treated groups and the metformin treated diabetic groups (Figure 1).

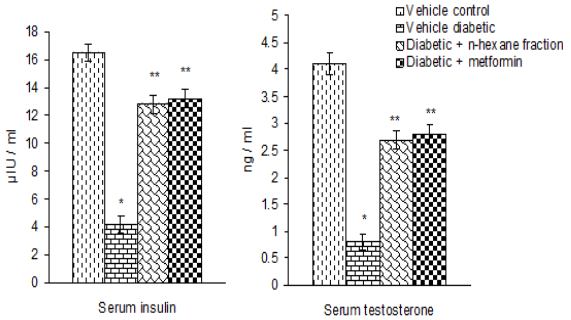


Figure 1. Effect of n-hexane fraction of hydro-methanol (2:3) extract of the *S. mahagoni* seeds on serum insulin and testosterone levels of STZ-induced diabetic male albino rats. All values are expressed as mean ± SD, n =6. Analysis of variance followed by multiple comparisons using the two-tailed Student-t test, where *indicates $P< 0.001$ and **indicates $P <0.05$ compared with vehicle control group.

3.4. Sperm Count, Viability and Motility

The epididymal sperm count and viability and motility were decreased significantly in the STZ-induced diabetic conditions when compared to the match vehicle control animals. Treatment of the diabetic animals with the n-hexane fraction resulted in significant ($p<0.05$) recovery of the above-mentioned parameters towards the control. The sperm count was recovered to the control level after the treatment of the diabetic animals with metformin though the sperm viability and motility were not recovered in this stage. No significant deviations were noted in sperm viability and motility between the n-hexane fraction-

treated group and the metformin treated diabetic groups (Table 3).

Table 3. Corrective role of *n*-hexane fraction of hydro-methanol (2:3) extract of the *S. mahagoni* seeds on sperm count, viability and motility in streptozotocin-induced diabetic male albino rats.

Groups	Sperm count (million/ml of epididymal fluid)	Sperm viability		Sperm motility (%)
		Alive sperm (%)	Dead sperm (%)	
Vehicle control	18.3±0.81	78.66±2.06	21.34±1.32	72.08±2.27
Vehicle diabetic	5.72±0.58*	33.51±1.87*	66.59±2.31*	41.71±1.92*
Diabetic +*n*-hexane fraction	13.2±0.69**	67.17±2.51**	30.83±1.7**	62.96±2.48**
Diabetic + metformin	17.8±0.76	68.47±2.63**	32.03±1.57**	64.17±2.61**

All values are expressed as mean ± SD, n = 6. Analysis of variance followed by multiple comparisons using the two-tailed Student-t test, where *indicates $P < 0.001$ and **indicates $P < 0.05$ compared with vehicle control group.

3.5. Activities of Testicular Δ5, 3β- Hydroxysteroid Dehydrogenase (Δ5, 3β- HSD) and 17 β- Hydroxysteroid Dehydrogenase (17 β- HSD) Enzymes

The testicular Δ^5, 3β- HSD and 17 β- HSD enzyme activities showed significant diminution in the vehicle diabetic rats when compared to the vehicle control animals. Activities of the above-mentioned enzymes in the testis were significantly recovered towards control after the treatment of the diabetic animals with n-hexane fraction. The 17 β- HSD enzyme activity was recovered to the control level after the metformin-treatment of the diabetic animals though the Δ^5, 3β- HSD enzyme activity was not recovered at this stage. There was no significant difference in the testicular Δ^5, 3β- HSD enzyme activity between the n-hexane fraction -treated groups and the metformin- treated diabetic groups (Figure 2).

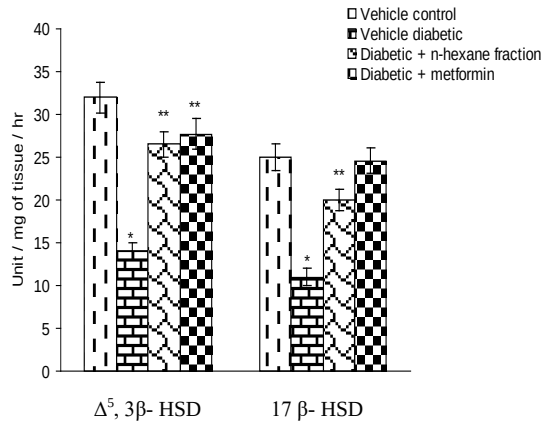


Figure 2. Corrective role of n-hexane fraction of hydro-methanol (2:3) extract of the *S. mahagoni* seeds on testicular Δ^5, 3β- HSD and 17β- HSD activities of STZ-induced diabetic male albino rats. All values are expressed as mean ± SD, n =6. Analysis of variance followed by multiple comparisons using the two-tailed Student-t test, where *indicates $P < 0.001$ and **indicates $P < 0.05$ compared with vehicle control group.

3.6. Testicular CAT, SOD and GST Enzymes Activities

Activities of CAT, SOD and GST in the testis were decreased significantly in vehicle diabetic animals in comparison to the vehicle control animals. After the n-hexane fraction treatment of the diabetic animals, the activities of the above-mentioned parameters were significantly recovered towards the control when comparison was made with the vehicle diabetic group. Metformin treatment of animals with diabetes resulted in resettlement of SOD activity to the control level whereas the CAT and GST activities were not recovered in this concern. Insignificant differences were noted in the activities of CAT and GST when a comparison was made between the n-hexane fraction treated groups, and the metformin treated diabetic groups (Figure 3).

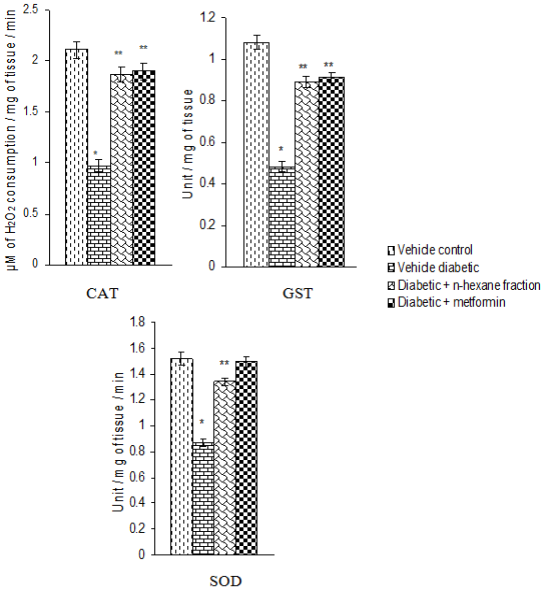


Figure 3. Remedial effect of *n*-hexane fraction of hydro-methanol (2:3) extract of the *S. mahagoni* seeds on testicular CAT, GST and SOD activities of STZ-induced diabetic male albino rats. All values are expressed as mean ± SD, n =6. Analysis of variance followed by multiple comparisons using the two-tailed Student-t test, where *indicates $P < 0.001$ and **indicates $P < 0.05$ compared with vehicle control group.

3.6. Levels of Conjugated Diene and Thiobarbituric Acid-Reactive Substances in Testis

Levels of testicular conjugated diene (CD) and thiobarbituric acid-reactive substances (TBARS) were significantly increased in the vehicle diabetic animals compared to the vehicle control animals. The treatment of the diabetic animals with n-hexane fraction or metformin at the above-mentioned dose resulted in significant recovery in the levels of these parameters towards the control. No significant deviations were noted in the levels of testicular CD and TBARS between the n-hexane fraction-treated groups and the metformin-treated diabetic groups (Figure 4).

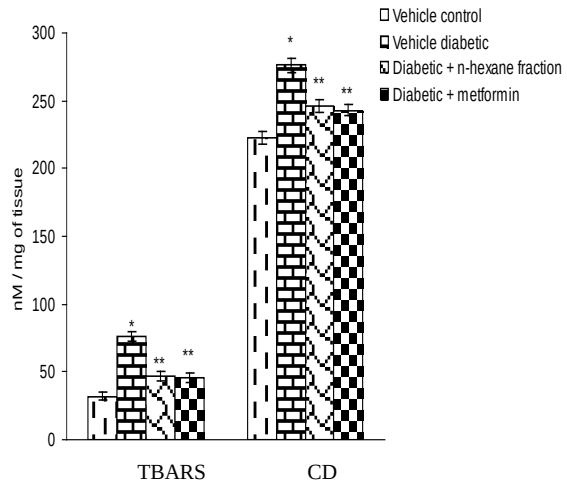


Figure 4. Effect of *n*-hexane fraction of hydro-methanol (2:3) extract of the *S. mahagoni* seeds on testicular TBARS and CD levels of STZ-induced diabetic male albino rats. All values are expressed as mean ± SD, n =6. Analysis of variance followed by multiple comparisons using the two-tailed Student-t test, where *indicates $P < 0.001$ and **indicates $P < 0.05$ compared with the vehicle control group.

3.7. Activities of SGOT and SGPT

Activities of transaminase enzymes, i.e., SGOT and SGPT, were increased significantly in vehicle diabetic animals in comparison with the vehicle control animals. After the treatment of the diabetic animals with n-hexane fraction or metformin at the above-mentioned dose in a fasting state, there was a substantial decrease in the activities of SGOT and SGPT towards the control level (Figure 5).

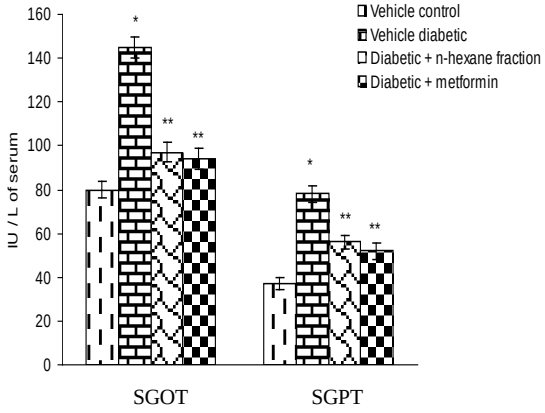


Figure 5. Corrective effect of *n*-hexane fraction of hydro-methanol (2:3) extract of the *S. mahagoni* seeds on serum GOT and GPT activities of STZ-induced diabetic male albino rats. All values are expressed as mean ± SD, n =6. Analysis of variance followed by multiple comparisons using the two-tailed Student-t test, where *indicates $P < 0.001$ and **indicates $P < 0.05$ compared with vehicle control group.

3.8. Histopathology of Pancreas

The diameters of pancreatic islets, count of islets, and islet cells per islet were substantially decreased. In addition, degeneration of pancreatic acini was also noted in the STZ-induced diabetic group in comparison with the vehicle control animals. The treatment of the diabetic animals with n-hexane fraction or metformin resulted in marked recovery of these parameters toward the control level. No significant deviations were noted in the above-mentioned parameters between the *n*-hexane fraction treated groups and the metformin-treated diabetic groups (Table 4 and Figure 6).

Table 4. Corrective effect of *n*-hexane fraction of hydro-methanol (2:3) extract of the *S. mahagoni* seeds on islet number, islet cell number, diameter of islets in streptozotocin-induced diabetic male albino rats.

Groups	Islet number (count per field in 1000X magnification)	No. Islet cells / Islet	Islet diameter (μ m)
Vehicle control	7.41±0.17	126.32±4.77	234.07±4.62
Vehicle diabetic	1.65±0.11*	71.74±3.38*	162.41±3.48*
Diabetic +*n*-hexane fraction	4.92±0.15**	106.59±4.32**	208.53±4.17**
Diabetic + metformin	5.06±0.16**	109.15±4.63**	210.76±3.95**

All values are expressed as mean ± SD, n = 6. Analysis of variance followed by multiple comparisons using the two-tailed Student-t test, where *indicates $P < 0.001$ and **indicates $P < 0.05$ compared with vehicle control group.

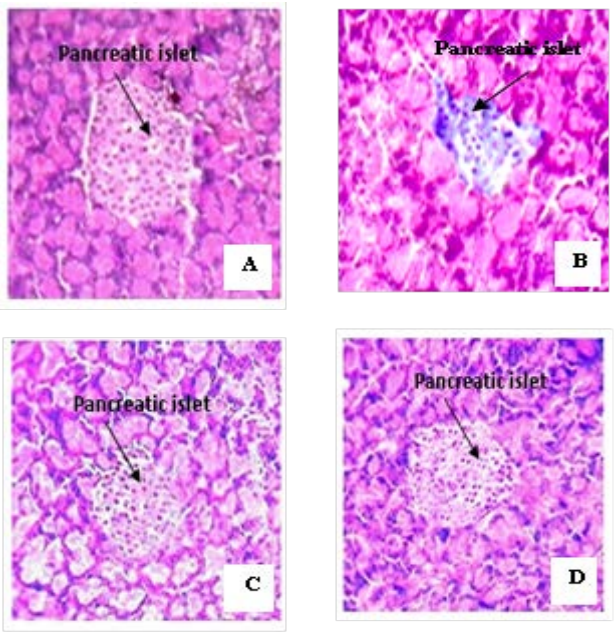


Figure 6. Histology of pancreas. **A:** Normal histoarchitecture of pancreatic islet, i.e. normal vehicle control group. **B:** Diminution in the diameter of the islet and islet cell population and degeneration of pancreatic acini cells in the vehicle diabetic group. **C and D:** Recovery of islet cell population and islet's diameter in n-hexane fraction-treated groups and metformin-treated diabetic groups (Haematoxylin-Eosin stain, X 400).

3.9. Histopathology of Testis

Vehicle treated control animals exhibited a normal histomorphological structure of testis, and dense lining of germ cells was noted in the periphery as well as in the middle part of the testis at stage-VII of the seminiferous epithelial cycle with normal diameter. Numbers of SgA, pLSc, mPSc, and 7Sd were all decreased significantly at

stage VII of the seminiferous epithelial cycle in the STZ-induced diabetic conditions in comparison to the vehicle control animals. The numbers of germ cells at stage VII of the seminiferous epithelial cycle were significantly recovered towards the control level after the n-hexane fraction treatment of the diabetic animals. Insignificant differences were noted in the numbers of SgA, pLSc, mPSc, and 7Sd germ cells at stage-VII of the spermatogenic cycle when comparisons were made between the *n*-hexane fraction treated groups and the metformin-treated diabetic groups (Table 5 and Figure 7).

Table 5. Effect of n-hexane fraction of hydro-methanol (2:3) extract of the *S. mahagoni* seeds on different generations of germ at stage VII in streptozotocin-induced diabetic male albino rats.

Groups	SgA	pLSc	mPSc	7Sd
Vehicle control	0.60±0.04	20.47±0.42	24.68±0.47	67.42±3.7
Vehicle diabetic	0.33±0.02*	9.75±0.32*	11.52±0.38*	34.05±2.8*
Diabetic +*n*-hexane fraction	0.53±0.03**	15.63±0.37**	19.27±0.40**	52.96±3.3**
Diabetic + metformin	0.54±0.04**	16.05±0.40**	19.86±0.45**	54.02±3.8**

All values are expressed as mean ± SD, n = 6. Analysis of variance followed by multiple comparisons using the two-tailed Student-t test, where *indicates $P < 0.001$ and **indicates $P < 0.05$ compared with vehicle control group.

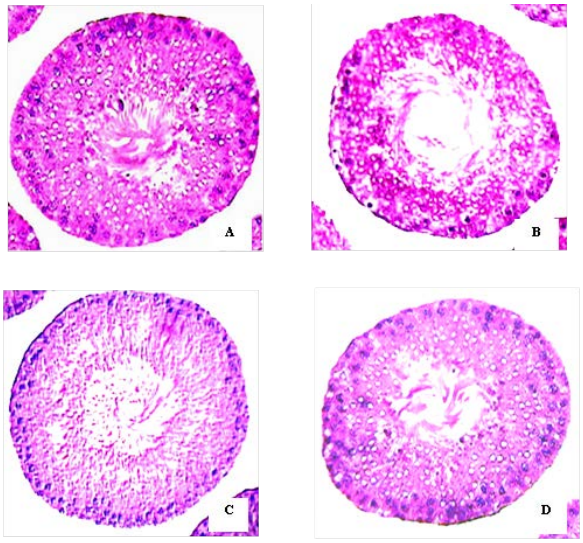


Figure 7. Histology of Testis. **A:** Normal density of different generations of germ cells in vehicle control group; **B:** Diminution in the number of different generations of germ cells at stage VII of vehicle diabetic group; **C and D:** Recovery in the number of different generations of germ cells at stage VII of n-hexane fraction and metformin-treated diabetic groups (Haematoxylin-Eosin stain, X 400).

3.10. Phytoingredients of n-Hexane Fraction

The *n*-hexane fraction of the hydro-methanol (2:3) extract from the seeds of *Swietenia mahagoni* (L.) Jacq. revealed the presence of medicinally-active antidiabetic and antioxidative constituents. The alkaloid, flavonoid and steroid constituents were present within the investigated fraction while terpenoid, and tannin were absent (Table 6).

Table 6. Phyto-constituents of *n*-hexane fraction of aqueous-methanol (2:3) extract of the sseds of *S. mahagoni* (L.) Jacq.

Phytoingredient(s)	*Swietenia mahagoni* (L.) Jacq.
Alkaloid	+
Flavonoid	+
Steroid	+
Terpenoid	-
Tannin	-

'+' indicate the presence of constituent and '–' indicate the bsence of constituent

4. Discussion

Diabetes mellitus (DM) is a leading disease of public health significance, which causes cardiovascular, psychological, and sexual dysfunctions. It is a well-known cause of male sexual dysfunction (MSD), with prevalence rates approaching 50% of both type 1 and type 2 diabetes mellitus (Johannes *et al.*, 2000). Day by day, the prevalence of MSD in diabetic patients is increasing. The incidences of impotence in diabetics are 2-5 times higher than non-diabetics (Zimmet, 2001). Impaired glucose metabolism leads to oxidative stress and protein glycation that in turn leads to a free radical generation. Diabetes suppresses the reproductive function resulting in male and female infertility (Rehman *et al.*, 2001; Enzlin *et al.*, 2002). Our data showed abnormal spermatogenesis following the STZ injection in male rats, which is concordant with data by other authors (Rabbani *et al.*, 2010). Our observations illustrate depressed sperm count and motility along with an increase in the percentage of dead sperm in diabetic animals. The number of different generations of germ cells at stage VII of the spermatogenic cycle was decreased significantly in diabetic rats in comparison to the control rats. This finding suggests that the conversion of spermatogonia into primary spermatocytes is reduced in diabetic conditions. These alterations in cellular conversion and/or activity lead to a reduction in the spermatozoid production. The current study was undertaken to investigate the remedial effect of the n-hexane fraction of the hydro-methanol (2:3) extract of *S. mahagoni* seeds on the management of diabetes-induced testicular dysfunctions noted in diabetic conditions. The effect was compared with a standard antidiabetic drug, namely, metformin.

The n-hexane fraction of the plant used here exhibited the most effective antidiabetic potentiality than other solvent fractions (i.e. chloroform, ethyl acetate, and n-butanol), supported by the study's report (Bera *et al.*, 2015). After the streptozotocin injection, the fasting blood glucose level increased and serum insulin levels were decreased; these results are in accordance with the authors' previous work (Bera *et al.*, 2015). After the treatment with the n-hexane fraction, there was a significant recovery in the levels of fasting blood glucose and serum insulin which can be attributed to the regeneration of the pancreatic β cells as confirmed by the present study and an earlier report by the same authors (Mallick *et al.*, 2007). Streptozotocin-induced diabetes resulted in significant diminution in testiculo-somatic, epididymal-somatic and

seminal vesiculo-somatic indices which may be attributed to the low- serum level of the testosterone because testosterone is the key regulator of the normal growth of these organs (Shrilatha and Muralidhara, 2007). After the treatment with this n-hexane fraction, the above-mentioned organo-somatic indices were recovered towards the control level which can be attributed to the elevation in serum testosterone. This elevation in serum testosterone after the administration of this fraction may be attributed to the elevation in the activities of androgenic key-enzymes i.e., Δ5, 3β-HSD and 17β-HSD, as well as by the recovery of serum insulin because insulin has a positive role in the testicular testosterone synthesis (Baccetti *et al*., 2002). This fraction may operate the proper tuning system of the pituitary-testicular axis by the insulin-glucose axis as proposed by others (Glenn *et al.*, 2003). Moreover, this fraction has the property to regenerate pancreatic β-cells; thus insulin levels will also increase and stimulate the testicular androgenesis. The n-hexane fraction has no general toxic effects reflected here by the body weight recovery and the measurement of the activities of SGOT and SGPT (Ghosh and Suryawanshi, 2001).

Another explanation for the corrective role of this *n*-hexane fraction on testis may be the correction in testicular oxidative stress noted here in the diabetic conditions by the measurement of testicular CAT, SOD and GST activities, and the important scavenging enzymes of reactive oxygen species (ROS) (Pillai and Gupta, 2005), in addition to the quantification of testicular TBARS and CD, which are the free radicals and end product (De-Young *et al*., 2004). The sperm count, which was decreased in diabetic states, may be due to the inhibition in spermatogenesis reflected here by the quantification of different generations of germ cells at stage VII of the spermatogenic cycle, an important reflector of holistic approach of spermatogenesis (Holstein *et al.*, 2003). Another possible reason behind the low sperm count is the effect of testicular oxidative stress on diabetic conditions reported by (Vincent *et al.*, 2002). Sperm motility and viability are also affected by the oxidative damaging effect of free radicals (De-Young *et al*., 2004). Increased cellular oxidative stress and altered antioxidant pool have been implicated in the pathogenesis of the chronic complications of diabetes (Vincent *et al*., 2002).

To comprehend the exact effect of oxidative stress on germ cells maturation and function, it is important to emphasize that some studies indicated the presence of high inducible cytochrome P-4502E1 isoform (CYP2E1) in male gonads (Healy *et al.*, 1999; Oropeza-Hernandez *et al.*, 2003; Quintans *et al.*, 2005). It is known that the CYP2E1 expression is affected by a variety of patho-physiological situations including diabetes (Knockaert *et al.*, 2011). Elevated oxidative stress and ROS production in diabetes often parallel an increased expression of CYP2E1 (Raza *et al.*, 2004). CYP2E1 generates reactive oxygen intermediates, such as superoxide radicals, which in turn could rapidly react with organic molecules generating secondary free radicals and ROS (Lieber, 1997). Such cascades may alter the antioxidant milieu of testis and epididymis; producing conditions for the spermatogenic cycle to interfere. After the administration of *n*-hexane fraction as protection from the testicular oxidative stress imposed in STZ-induced diabetic conditions, the sperm count, and viability, as well as a number of different generations of germ cells at stage VII were corrected towards the control level. This antioxidant activity of the n-hexane fraction may be due to the presence of phytoingredients such as alkaloid, flavonoid, and steroid which are traditional natural antioxidants.

5. Conclusion

From the preceding discussion, it may be concluded that this *n*-hexane fraction has a significant protective effect on testicular dysfunctions noted in STZ-induced diabetic conditions. The exact chemical compound(s) of n-hexane fraction responsible for such protection remains speculative, needing further studies to isolate, identify, and characterize the active ingredients and their molecular action in this regard.

Conflict of Interest Statement

We declare that we have no conflict of interest

References

Abercrombie M. 1947. Estimation of nuclear population from microtome sections. *Anat Record,* **99**: 239-247.

Baccetti B, La Marca A, Piomboni P, Capitani S, Bruni E and Petraglia F. 2002. Insulin-dependent diabetes in men is associated with hypothalamo-pituitary derangement and with impairment in semen quality. *Human Reprod.,* **17**: 2673-2677.

Beers RF and Sizer IW. 1952. Spectrophotometric method for measuring the breakdown of hydrogen peroxidase by catalase. *J Biol Chem.,* **195**: 133-140.

Bera TK, Chatterjee K and Ghosh D. 2015. Remedial hypoglycemic activity of n-hexane fraction of hydro-methanol extracts of seed of *Swietenia mahagoni* (L.) jacq. in streptozotocin-induced diabetic rat: A comparative evaluation. *J Herbs Spices Medicinal Plant,* **21**: 38-58.

Bera TK, De D, Chatterjee K, Ali KM and Ghosh D. 2010. Effect of Diashis a polyherbal formulation in streptozotocin-induced diabetic male albino rat. *Int J Ayurveda Res.,* **1**: 18-24.

Briner M, Brugi W, Fraken N and Kessler ACH. 1988. One step sandwich enzyme immunoassay for insulin using monoclonal antibodies. *Clin Biochem.,* **21**: 311-314.

Brüning JC, Gautam D, Burks DJ, Gillette J, Schubert M, Orban PC, Klein R, Krone W, Müller-Wieland D and Kahn CR. 2000. Role of brain insulin receptor in control of body weight and reproduction. *Science*, **289**: 2122-2125.

Bucholtz DC, Chiesa A, Pappano WN, Nagatani S, Tsukamura H, Maeda KI and Foster DL. 2000. Regulation of pulsatile luteinizing hormone secretion by insulin in the diabetic male lamb. *Biol Reprod.,* **62**:1248-1255.

Can A, Akev N, Ozsoy N, Bolkent S, Arda BP, Yanardag R and Okyar A. 2004. Effect of *Aloe vera* leaf gel and pulp extract on the liver in type II diabetic rat models. *Biol Phrm Bull*., **27**: 694-698.

Clermont Y and Morgentaler H. 1955. Quantitative study of spermatogenesis in hypophysectomized rat. *Endocrinol.,* **57**: 369-382.

De-Young L, Yu D, Bateman RM and Brock GB. 2004. Oxidative stress and antioxidant therapy: their impact in diabetes-associated erectile dysfunction. *J Androl.,* **25**: 830-836.

Edeoga HO, Okwu DE and Mbaebie BO. 2005. Phytochemical constituents of some Nigerian medicinal plants. *African J Biotechnol.*, **4**: 685-688.

Enzlin P, Mathieu C, Bruel A and Bosteels D. 2002. Sexual dysfunction in women with type I diabetes: a controlled study. *Diabetes Care*, **25**: 672-677.

Ghosh S and Suryawanshi SA. 2001. Effect of *Vinca rosea* extracts in treatment of alloxan diabetes in male albino rats. *Ind J Exp Biol.*, **39**: 748-759

Glenn DR, McClure N and Lewis SE. 2003. The hidden impact of diabetes on male sexual dysfunction and fertility. *Human Ferti (Camb).*, **6**: 174-179.

Gnoth C, Godehardt E, Frank-Herrmann P, Friol K, Tigges J and Freundl G. 2005. Definition and prevalence of subfertility and infertility. *Human Reprod.*, **20**: 1144-1147.

Healy LN, Puta LJ and Recio L. 1999. Expression and distribution of cytochrome P450 2E1 in B6C3F1 mouse liver and testes. *Chem Biol Interact.*, **121**:199-207.

Henry RJ, Chiamori M, Golub OJ and Berkman S. 1960. Revised spectrophotometric methods for the determination of glutamate oxaloacetic transaminase, glutamic pyruvate transaminase and lactic acid dehydrogenase. *Amer J Clin Pathol.*, **34**: 381-398.

Hobig WH, Pabst MJ and Jakoby WB. 1974. Glutathione-S-transferase. The first enzymatic step in mercapturic acid formation. *J Biol Chem.*, **249**: 7130-7139.

Holstein AF, Schulze W and Davidoff M. 2003. Understanding spermatogenesis is a prerequisite for treatment. *Reprod Biol Endocrinol.*, **14**:1-16.

Jaraback J, Adams JA, Williams-Ashman HG and Talalay P. 1962. Purification of a 17β-hydroxysteroid dehydrogenase of human placenta and studies on its transhydrogenase function. *J Biol Chem.*, **237**: 345-357.

Johannes CB, Araujo AB, Feldman HA, Derby CA, Kleinman KP and McKinlay JB. 2000. Incidence of erectile dysfunction in men 40 to 69 years old: longitudinal results from the Massachusetts male aging study. *J Urol.*, **163**: 460-463.

Kirk TK. 2009. **Tropical Trees of Florida and the Virgin Islands: A guide to Identification, Characteristics and Uses.** Pineapple Press Inc. ISBN 978-1-56164-445-2. p.144.

Knockaert L, Fromenty B and Robin MA. 2011. Mechanisms of mitochondrial targeting of cytochrome P4502E1: physiopathological role in liver injury and obesity. *FEBS J.*, **278**: 4252-4260.

Kolawole OM, Oguntoye SO, Agbede O and Olayemi AB. 2006. Studies on the efficacy of *Bridella ferruginea* Benth. bark extract in reducing the coliform load and BOD of domestic waste water. *Ethnobot Leaflets*, **10**: 228-238.

Leblond CP and Clermont Y. 1952. Definition of the stage of the cycle of seminiferous epithelium in the rat. *Annual N Y Acad Sci.*, **55**: 548-573.

Li DD, Chen JH, Chen Q, Li GW, Chen J and Yue JM. 2005. *Swietenia mahagoni* extract shows agonistic activity to PPAR (gamma) and gives ameliorative effects on diabetic db/ db mice. *Acta Pharmacol Sin.*, **26**: 220-222.

Lieber CS. 1997. Cytochrome P-4502E1: its physiological and pathological role. *Physiol Rev.*, **77**: 517-544.

Mallick C, Bera TK, Ali KM, Chatterjee K and Ghosh D. 2010. Diabetes induced vis-à-vis germ cells apoptosis in albino rats: Remidial effects of n-hexane fraction of root of *Musa paradiciaca* and leaf of *Coccinia indica*. *J Health Sci*., **56**: 641-654.

Mallick C, Chatterjee K, Mandal U and Ghosh D. 2007. Antihyperglycaemic, antilipiperoxidative and antioxidative effects of extracts of *Musa paradisiaca* and *Coccinia indica* in streptozotocin-induced diabetic rat. *Ethiop Pharm.*, **25**: 9-22.

Mallick C, De D and Ghosh D. 2009. Correction of protein metabolic disorders by composite extract of *Musa paradisiaca* and *Coccinia indica* in streptozotocin-induced diabetic albino rat. *Pancreas*, **38**: 322-329.

Marklund S and Marklund G. 1974. Involvement of superoxide anione in autooxidation of pyrogallol and a convenient assay of superoxide dismutase. *Eur J Biochem.*, **47**: 469-474.

Okhawa H, Ohishi N and Yagi K. 1979. Assay for lipid peroxidation in animal tissues thiobarbituric acid reaction. *Anal Biochem.*, **95**: 351-358.

Oropeza-Hernandez LF, Quintanilla-Vega B, Reyes-Mejia RA and Serrano JC. 2003. Trifluoroacetylated adducts in spermatozoa, testes, liver and plasma and CYP2E1 induction in rats after subchronic inhalatory exposure to halothane. *Toxicol Letter*, **144**: 105-116.

Pakarainen T, Zhang FP, Mäkelä S, Poutanen M and Huhtaniemi I. 2005. Testosterone replacement therapy induces spermatogenesis and partially restores fertility in luteinizing hormone receptor knockout mice. *Endocrinol.*, **146**:596-606.

Panda DK, Ghosh D, Bhat B, Talwar SK, Jaggi M and Mukherjee R. 2009. Diabetic therapeutic effect of ethyl acetate fraction from the roots of *Musa paradisiaca* and seeds of *Eugenia jambolana* in streptozotocin-induced male diabetic rat. *Methods Find Exp Clin Pharmacol.*, **31**: 571-584.

Pari L and Umamaheswari J. 2000. Antihyperglycaemic activity of *Musa sapientum* flowers: effect on lipid peroxidation in alloxan diabetic rats. *Phytother Res.*, **14**: 1-3.

Pillai A and Gupta S. 2005. Antioxidant enzyme activity and lipid peroxidation in liver of female rats co-exposed to lead and cadmium: effects of vitamin E and Mn^{2+}. *Free Rad Res.*, **39**: 707-712.

Quintans LN, Castro GD and Castro JA. 2005. Oxidation of ethanol to acetaldehyde and free radicals by rat testicular microsomes. *Archive Toxicol.*, **79**: 25-30.

Rabbani SI, Devi K and Khanam S. 2010. Pioglitazone, a PPAR-γ ligand inhibited the nicotinamide-streptozotocin-induced sperm abnormalities in type-2 diabetic Wistar rats. *Pakistan J Pharm Sci.*, **23**: 326-331.

Raza H, Pradu SK, Robin MA and Avadhani NG. 2004. Elevated mitochondrial cytochrome P450 2E1 and glutathione S-transferase A1-4 in streptozotocin-induced diabetic rats: tissue-specific variations and roles in oxidative stress. *Diabetes*, **53**: 185-194.

Rehman K, Beshay E and Carrier S. 2001. Diabetes and male sexual function. *Sex Repro Med.*, **1**: 29-33.

Shrilatha B and Muralidhara S. 2007. Early oxidative stress in testis and epididymal sperm in streptozotocin-induced diabetic mice: its progression and genotoxic consequences. *Repro Toxicol.*, **23**: 578-587.

Sicree R, Shaw J and Zimmet P. 2006. Prevalence and projections. In: Gan D (Ed.), **Experimental Model of Diabetes**. Atlas international diabetes federation, Belgium, pp. 16-104.

Sing S, Yuvaraj S, Malini T and Balasubramanian K. 2005. Experimental diabetes has adverse effects on the differentiation of ventral prostate during sexual maturation of rats. *Discov Mol Cell Evol Biol*., **287**: 1281-1289.

Slater TI. 1984. Overview of methods used for detecting lipid peroxidation. *Methods Enzymol.*, **105**: 283-293.

Sokal RR and Rohle FJ. 1997. Introduction to analysis of variance. In: Sokal RR and Rohle FJ (Eds.), **Biometry**. WH Freeman and Company, New York, pp.179-206.

Soudamani S, Malini T and Balasubramanian K. 2005. Effects of streptozotocin- diabetes and insulin replacement on the epididymis of prepubertal rats: histological and histo-morphometric studies. *Endocr Res.*, **31**: 81-98.

Srivastava TG. 2001. Orientation training course on research methodology in reproductive medicine. Department of Reproductive Medicine, National Institute of Health and Family Welfare (NIHFW), New Delhi, pp. 55-58.

Talalay P. 1962. Hydroxysteroid dehydrogenase. In: Colowick SP and Kaplan NO (Eds.), **Methods in Enzymology**. Academic Press, New York, pp. 512-516.

Trease GE and Evans WC. 2002. **Pharmacognosy**, fifth ed. Bailliere Tindall Ltd, London.

Vincent AM, Brownlee M and Russell JW. 2002. Oxidative stress and programmed cell death in diabetic neuropathy. *Annual N Y Acad Sci*., **959**: 368-383.

WHO study group. 2002. Diabetes mellitus: Technical report series. World health organization, Geneva, pp. 844.

World Health Organization. 1999. Laboratory manual for the examination of human semen and sperm-cervical mucus interaction. 4th ed. Cambridge University Press, New York, pp. 68-70.

World Health Organization. 2001. Legal status of traditional medicines and complementary/alternative medicines: a worldwide review. WHO, Geneva, (WHO/EDM/TRM/2001.2).

Zimmet P, Albert K and Shaw J. 2001. Global and societal implications of the diabetes epidemic. *Nature*, **414**: 782-787.

JJBS

Volume 11, Number 2,June 2018
ISSN 1995-6673
Pages 141 - 145

Jordan Journal of Biological Sciences

Physiological Indications and Gut-Microbial Community in Army Personnel in High- Altitude and Base-Line Environments: A Comparative Study

Animesh Samanta[1], Arpita Patra[1], Arpita Mandal[1], Suchismita Roy[1], Shreya Mandal[1], Koushik Das[1], Banadeb Sinha[2], Sanjay Kar[3] and Dilip K Nandi[1,*]

[1]*Department of Microbiology, Nutrition and Human Physiology, Raja N L Khan Women's College, Midnapore, Pin-721102,*

[2]*Department of Biochemistry, Midnapore Medical College and Hospital, West Bengal, India.*

[3]*Department of Botany, Midnapur College, Midnapore, Pin-721102, West Bengal, India.*

Received July 4, 2017; Revised September 9, 2017; Accepted October 26, 2017

Abstract

High altitude (HA) environments contain low levels of oxygen, which often cause nonspecific gastrointestinal (GI) disorders associated with acute mountain sickness (AMS). This study is conducted to investigate the alternation of microbial population and the physiological indices after being exposed to HA. Fecal and blood samples were collected from twelve army personnel in base-level environments in (Delhi, India) after acclimatization for seven days at 3505 m HA (Lah, India). Different bacterial groups, oxygen saturation level (SPO_2), serum hemoglobin (Hb), hematocrit (HCT), blood urea, creatinine and microbial enzymes such as the α-amylase, proteinase, alkaline phosphatase and β-glucuronidase levels were all measured. It was observed that the total aerobic bacteria decreased significantly and the anaerobic and facultative anaerobic increased gradually in the intestines. Strict anaerobes including *Bacteroidetes* sp*., Bifidobacterium* sp*.*, *Lactobacillus* sp. as well as pathogenic bacteria such as the *Salmonella* sp. exhibited positive growth. Various physiological parameters including serum hemoglobin, hematocrit, and urea-creatinine were also significantly changed. The microbial enzyme- activity also increased at HA. This confirms that the exposure to HA can change the intestinal microbial population affecting the microbial enzyme production possibly causing GI dysfunctions as a result of the decreased availability of oxygen.

KeyWords*:* High-altitude (HA); Acute mountain sickness (AMS); Microbial enzymes; Gut-microflora; Oxygen saturation level (SPO_2)**.**

1. Introduction

Hypobaric hypoxia is a characteristic environmental condition in high-altitude areas where people are known to develop varieties of physiological difficulties in such conditions (Hackett and Roach 2001). The stresses are solely dependent on the rise of altitude from sea level [an increase of 1 km above sea level drops 10 kilopascal (kPa) air pressure; with a sea level air pressure of 101.3 kPa (at 15 ℃ and 0% humidity)] (Hackett and Roach 2001). At high altitude, individuals may suffer from several patho-physiological disorders including a change in body weight, hematological changes, gastrointestinal disorders which are collectively called the altitude related sickness (ARS) (Shaov and Wan 2005; Paula and Niebauer 2012). Acute mountain sickness (AMS) is a frequent complication of individuals at high altitudes (Anand *et al.* 2006). One of the important problems in AMS is the gastrointestinal disorders that consist of indigestion, acid formation, flatulence, vomiting, anorexia, and diarrhea (Rook and Brunet 2005).

It is well-known that the normal human gastrointestinal (GI) tract contains vast and diverse groups of microbes in a complex ecosystem consisting of nearly one-hundred trillion (over fifty bacterial phyla; about 500 to 1,000 bacterial species). On the whole, these bacteria are very active, and play a significant role in diseases and human health (Hao and Lee 2004). Any responses of the host inside the state of an exacting range of exogenous factors (stress, temperature, drugs, cancer, etc.) and endogenous factors (inflammatory bowel diseases, peristalsis disorders) can change the GI microenvironment as well as its microbial ecology (Rhee *et al.* 2009). A disproportion of GI microbial ecology affects the gut physiological homeostasis, and may induce GI problems (Ley *et al.* 2012). It was shown that at high altitude (HA), gastrointestinal disorders are common problems for soldiers, veterans, athletes and travellers. Clues behind these disorders have not yet been explored, and there is no

* Corresponding author. e-mail: dilipnandi2004@yahoo.co.in.

thorough evidence for variations in the GI microbial flora at high altitude. To analyze the microbial community of the GI tract, generally cultivable and non-cultivable approaches are used. The major drawback of cultivable approaches is that it reflects only a limited number of microbes (30-40%) of the whole microbial community. Now, the restriction of the cultivable process was minimized by the advance of modern molecular techniques (non-cultivable approach). However, the latter are very expensive, and so the cultivable method is still regarded as a classical technique (Lemos *et al.* 2012).

In the present study, quantitative variation of some common bacteria of fecal samples of healthy army male soldiers at a base level environment (Delhi), and at a HA environment (Leh-Ladakh) were examined. Microbial analysis including total aerobes and prominent anaerobes, an indicator strain *Escherichia coli*, Lactic acid bacteria, *Bifidobacterium* sp., *Bacteroidetes* sp. and a pathogenic strain *Salmonella* sp. were studied to investigate the impact of hypobaric hypoxia on the composition of gastrointestinal microbiota and some physiological indices. In relation to the microbial alteration, the activity of some microbial enzymes was also monitored.

2. Materials and Methods

2.1. Subject Selection

Twelve young healthy Indian army male soldiers (base level residents) within the age group of 25 – 30 years were selected for this study; their body mass index (BMI) was approximately 24.55 kg/m^2 (± 0.84). They all were healthy, not under the treatment of any medication, and they were not suffering from any bacterial or viral infections. They consumed army-specific homogenous diets throughout the experiment. The sea level (i.e. Base line or '0' day) study was carried out at Delhi (barometric pressure 740 mm Hg). After recording the physiological parameter and collecting the samples, the subjects were flown to an altitude of 3,505 m at Leh (barometric pressure 483 mm Hg) in the Western Himalayas. The subjects arrived at a 3500 m altitude in the morning and the day of arrival was taken as day one at 3500 m.

2.2. Collection of Fecal Samples

After a brief medical counseling, the fecal samples (~10 g) were collected in a pre-sterilized spatula–container at Delhi, and these were considered as the 'Base Line' (or '0' day sample) samples. Thereafter, the samples were collected on the first, fourth, and the seventh days at Leh, Jammu and Kashmir, India (~ 3500 m) during acclimatization. The samples were transported in a sterilized carrier solution containing peptone, 10 % (w/v); glycerol, 5 % (v/v). pH was adjusted to 7.0±0.2, and the samples were stored at 4 ºC until the analysis.

2.3. Collection of Blood Samples

Blood samples were collected from subjects using 21-Gauge needles (21G) mounted on a 5-mL syringe (Hindustan Syringes and Medical Devices Ltd, Faridabad, India) into heparin-coated sample bottles for analysis. Samples were collected on '0' (base line), during the first, fourth, and seventh day periods.

2.4. Analytical Measurement

2.4.1. Microbial Analysis

The cultivable microflora was enumerated on agar plates on the basis of colony-forming units (CFU). CFU represent the actual number of bacteria present in the fecal samples. These CFU values were converted to their logarithmic value and tallied with the corresponding experimental set of specified conditions. The total aerobic and anaerobic fecal bacteria were enumerated by a standard pour-plate technique in a single-strength trypticase soya agar (HiMedia, India), and reduced Wilkins Chalgren agar (supplemented with sodium succinate, hemin, vancomycin, menadione, oleandomycin phosphate polymyxin B and nalidixic acid) respectively. The Anaerobic condition was maintained in CO_2 incubator filled with 10% of CO_2 and H_2 gases (Adak *et al.*, 2013). *Escherichia coli, Bacteroidetes sp.,* total Lactic acid bacteria, *Bifidobacterium sp.,* and *Salmonella sp.* were cultured on Mac-Conkey, bacteroides bile esculin agar (supplemented with gentamicin 100 mg/L),De Man, Rogosa and Sharpe agar (MRS), bifidobacterium and Brilliant green agar modified (HiMedia, India) respectively (Wehr and Frank 2004; Maity *et al*., 2012).

2.4.2. Somatic Index and Haematological Parameter

The individual body weight, body temperature, and heart rate were recorded at the beginning (Base Line) and throughout the experiment on the first, fourth, and seven days. A finger pulse Oximeter probe (Model MU 300, China) was set on the right index finger to measure the pulse and oxygen saturation level (SPO_2). The blood samples were used for analyzing the hematological parameters including hemoglobin (Hb) by the standard kit method (Merck, Japan) and hematocrit (HCT).

2.5. Blood Uremia Profile

2.5.1. Biochemical Estimation of Blood Urea

The plasma fraction was separated after the centrifugation of the blood samples at 3,000 rpm for five minutes. Plasma urea levels were measured by the commercially available standard blood urea kit (Merck, Japan) following the standard protocol for photometric determination of urea according to the glutamate dehydrogenase method (Burtis and Ashwood 1999).

2.5.2. Biochemical Estimation of Blood Creatinine

Plasma creatinine levels were measured by the commercially available standard creatinine kit (Merck, Japan) following the standard protocol for photometric determination of creatinine according to the Jaffe kinetic method without deproteinization (Sabbagh *et al.* 1988).

2.6. Fecal Enzyme Activity

The fecal samples were centrifuged at 10,000 rpm for fifteen minutes at 4 ºC. for the enzymatic analysis. After centrifugation, the supernatant was collected and used in the enzyme assay. For the determination of α-amylase activity, the dinitro-salicyclic acid method was used (Miller 1989). Proteinase, alkaline phosphatase and β-glucuronidase activities were assayed by the following protocols of Brock *et al.* (1982), Yotton and Savage (1976) and Kent *et al.* (1972) with slight modifications. Total protein in the feces was determined by Lowry *et al.*

(1951). The enzyme activities were expressed as specific activity (U/mg of protein).

2.7. Statistical Analysis

The collected data are presented as the arithmetic mean of three replicas (mean ± SE). The variations in microbial count hematological parameters were examined by one-way ANOVA. The alteration in the bacterial quantity was tested by Bonferroni for post hoc testing. Significant variation was accepted at the level of 5 %, i.e. $p<0.05$.

3. Results

A large number of aerobic bacteria were present in the fecal samples at normobaric conditions. But it was reduced significantly ($p<0.05$) after the seventh day of acclimatization at Leh. The quantity of total anaerobes was 9.10 ($\log_{10}^{CFU/g}$) on the 0^{th} day at base line (Delhi) and increased significantly to 11.15 ($\log_{10}^{CFU/g}$) after the seventh day of acclimatization. The ratio of total anaerobe to aerobic bacteria was 10^3 in Dehli; it had increased to 10^7 on the seventh day at Leh. The *E. coli* content was 6.9 ($\log_{10}^{CFU/g}$) at the base level, it was expended after the seventh days acclimatization of hypoxic condition and the changes of the above population were statistically significant, with $p < 0.05$. The quantity of strict anaerobic such as *Bacteroides* sp. and *Bifidobacterium* sp. was increased after the seventh day (Table 1). The total lactic acid bacterial population was increased though it showed no significant ($p<0.05$) change up to the seventh day of acclimatization. Selected pathogen such as Salmonella sp. was increased at the seventh day of acclimatization higher than the base line population. The body weight of the experiment group of the army personnel (AP) was decreased at the end of seventh day of acclimatization compared with their initial body weight; though it was not changed significantly ($p < 0.05$) (Table 2). Initially the body temperature (Table 2) and SPO_2 decreased (Table 2), during acclimatization it was further increased towards the normal. Heart rate (Table 2), the level of hemoglobin (Hb) and hematocrit (HCT) were significantly increased during acclimatization (Table 3).

The alteration of urea and creatinine levels were significant in group AP, it was noticed that during acclimatization (AP group) urea and creatinine levels were increased gradually and on the seventh day of hypoxic exposure they returned to the initial base line values (Table 3).

The microbial enzyme activity was also used for the evidence of the alternation of GI micro-environment during HA. The base levels of alkaline phosphatase, proteinase, β-glucuronidase and α-amylase activity increased respectively % in their specific activity after seven days of acclimatization (Table 4).

Table 1. Alteration of microbial population in human feces (Army Personnel - AP) on different days during acclimatization at high altitude (3500m).

MicrobialParameters	Hypobaric hypoxic exposure duration (in days)			
	'0' (Base Line)	1	4	7
Total aerobes	6.13 ± 0.554^a	6.30 ± 0.561^a	4.77 ± 0.548^b	3.94 ± 0.560^c
Total anaerobes	9.1 ± 0.581^b	9.10 ± 0.583^b	11.10 ± 0.584^a	11.15 ± 0.585^a
Escherichia coli	6.9 ± 0.482^c	6.97 ± 0.480^c	7.98 ± 0.483^b	8.95 ± 0.481^a
Bacteroidetes sp.	7.03 ± 0.275^c	7.20 ± 0.271^c	7.78 ± 0.700^b	8.23 ± 0.277^a
Total Lactic Acid Bacteria	6.3 ± 0.312^b	6.34 ± 0.310^b	7.35 ± 0.313^a	7.45 ± 0.314^a
Bifidobacterium sp.	5.21 ± 0.450^b	5.38 ± 0.451^b	6.54 ± 0.453^a	7.08 ± 0.452^a
Salmonella sp.	2.26 ± 0.474^c	2.33 ± 0.470^c	3.52 ± 0.471^b	4.21 ± 0.472^a

*Microbial population density was expressed (mean of $\log_{10}$ CFU/g ± SD). Letters (a, b, c) in superscript form in the row are significantly different at $p<0.05$.

Table 2. Changes physiological parameter on different days of acclimatization to 3500m.

	Changes of physical parameter			
	Base Line	HA Day 1	HA Day 4	HA Day 7
Body Weight (kg)	69.5 ± 2.58	69 ± 2.67	66 ± 2.69	66 ± 2.65
Heart Rate (pulse/min)	64.83 ± 3.49	81.5 ± 7.46	84.33 ± 6.20	81.66 ± 6.41
SPO2 (%)	98.83 ± 0.23	91.66 ± 1.07	97.16 ± 0.43	97.83 ± 0.92
Temperature (ºF)	98.41 ± 0.21	96.3 ± 0.80	96.96 ± 0.62	97.06 ± 0.63

* Data are expressed as Mean ± SE.

Table 3. Changes of blood parameter of Army Personnel (AP) on different days of acclimatization and its alteration after seven days at 3500m.

Blood parameters	Groups	Hypobaric hypoxic exposure duration (in days)			
		'0' (Base Line)	1	4	7
Hb (g/dl)	AP	14.16 ± 0.52^{c}	16.1 ± 0.31^{a}	15.76 ± 0.48^{b}	15.84 ± 0.52^{b}
Hematocrit (HCT)	AP	50.8 ± 1.93^{a}	51.4 ± 1.71^{a}	48.4 ± 1.19^{b}	48 ± 1.41^{b}
Urea (mg/dl)	AP	33.16 ± 1.43^{c}	37.83 ± 3.37^{b}	43 ± 3.22^{a}	34.5 ± 3.21^{c}
Creatinine(mg/dl)	AP	0.83 ± 0.05^{c}	0.95 ± 0.08^{a}	1.06 ± 0.08^{a}	0.87 ± 0.04^{b}

*Data are expressed as Mean ± SE.Letters (a, b, c) in superscript form in the row are significantly different each other at $p<0.05$.

Table 4. Changes in the enzyme profiles on different days of acclimatization.

Enzyme activity	Hypobaric hypoxic exposure duration (Day)				
	'0' (Base Line)	1	2	4	7
A-amylase	200.22 ± 4.45^{d}	210.34 ± 4.38^{c}	222.22 ± 4.11^{b}	241.44 ± 5.05^{a}	244.57 ± 4.21^{a}
Proteinase	3.83 ± 0.06^{d}	4.05 ± 0.05^{c}	4.47 ± 0.02^{b}	5.82 ± 0.08^{a}	5.90 ± 0.1^{a}
β-glucuronidase	5.75 ± 0.06^{e}	6.04 ± 0.04^{d}	6.63 ± 0.03^{c}	7.82 ± 0.02^{b}	8.05 ± 0.05^{a}
Alkaline phosphatase	4.43 ± 0.01^{e}	5.12 ± 0.02^{d}	6.17 ± 0.02^{c}	6.67 ± 0.04^{b}	7.18 ± 0.1^{a}

* Data are expressed as Mean ± SE. Letters (a, b, c, d, e) in a row are significantly different each other at $p<0.05$.

4. Discussion

The population of microbes present in the microenvironment of the gastrointestinal tract performs several important and essential activities in the host body. Little disturbances in the balanced gastrointestinal environment result in the alteration of the whole ecosystem with resultant physiological changes (Rhee *et al.,* 2009). Acute exposure to high altitude, the individual suffers from AMS due to the decrease in inspired PO_2, while traveling from sea level. Acclimatization to high altitude decreases the tissue oxygen delivery, which causes microcirculatory dysfunctions and cellular dyslexia including indigestion, acid gas formation, bowel motility, permeability etc. This dyslexia in the gastrointestinal track (GI) mucosa leads to metabolic dysfunctions that finally have an effect on the largest number of GI symbionts.

The results of this study showed that the total aerobes of the fecal samples decreased significantly during acclimatization of army personnel at high altitude, and the total anaerobes increased after seven days of HA acclimatization. This is likely related to the higher anaerobic state of intestinal epithelia, and the alteration of GI mucosal microenvironment was the major factor causing the modulation of specific bacterial subpopulations.

It has been established that the *E. coli* population was generally 10^6 times higher than the total aerobes in feces. The total aerobes, facultative anaerobes (*E. coli*) and total anaerobes are present in the ratio of $4.36:1:4.03\times10^5$ in feces, but this may vary within species and even between individuals in the same species (Maity *et al*., 2009). At a lower level of oxygen, this ratio was changed to $1 : 2.94 \times 10^4 : 2.16 \times 10^7$ and *E. coli* proliferation was higher (10^6) as it possessed elaborate genetic regulatory network for sensing oxygen (Holy *et al.,* 2012). It has been shown that immobilization for six hours initiates the increase of the concentration of *E. coli* in the proximal sections (the duodenum and the jejunum) of the digestive tract (Gritsenko *et al.,* 2000). This rapid expansion of *E. coli* population may encourage the growth of other strict anaerobes (*Bacteroidetes* sp. *Bifidobacterium* sp. and *Lactobacillus* sp.*)* and pathogen (*Salmonella* sp.*)* in anaerobic respiration (Gombosov *et al.,* 2011). But it is not clear, why *Bacteroidetes* sp. and lactic acid bacteria were lower than other anaerobes. The growth of Gram negative bacteria can cause a serious burden in the gut lumen due to poisoning with bacterial lipopolysaccharides.

The loss of body weight at hypobaric hypoxic conditions had been described in several studies (Benso *et al.,* 2007). In the present study, the final body weight of the army personnel (AP) was not changed significantly during the seven-day period of the experiment, but there was a tendency to weight loss (Table 2) which may be attributed to the higher metabolic rate, different energy output, and the loss of body water (Wall *et al.,* 2009). Initially the body temperature and SPO_2 were decreased, and at the seventh day of acclimatization they were increased due to the lower oxygen concentration in the air. In such conditions, the heart rate altered significantly due to the activity of autonomic nervous system (Bhaumik *et al.,* 2013).

The level of hemoglobin and HCT were increased during acclimatization to HA. Literature reveled that hypoxia causes the excessive secretion of erythropoietin (EPO) to increase blood RBC and Hb (Wickler *et al.,* 2000; Mizuno *et al.,* 2008) in order to compensate for the reduced blood oxygen content. In the current study, blood Hb and HCT were increased (Table 2) as describes by (Mairbaurl 2013). It was known several decades ago that erythrocytes are produced from the successive maturations of different erythroid progenitors which are responsive to erythropoietin (EPO). The rise in HCT and hemoglobin contents suggests that HA increases the EPO production (EPO is a glycoprotein hormone produced by the kidneys and secreted into the plasma), producing then haematological changes, (Gouttebargea *et al.,* 2012).

The alkaline phosphatase activity removed the phosphate from glutamine of the lipid moiety to reduce the LPS toxicity and create a less toxic situation (Bates *et al.,* 2006). The α-amylase activity digests the undigested polysaccharides to salvage energy and facilitated acid accumulation in the colon (Gloster *et al.,* 2008).

This study shows that the lower pressure of atmospheric oxygen at high altitude reduced the blood oxygen level that disturbed the physiological buffering system. As a result, gut microbiota and its associated enzymes are altered, perhaps causing the indigestion and the GI complications during the acclimatization at HA.

Acknowledgement

The authors are thankful to the Director of the Defense Institute of Physiology & Allied Sciences (DIPAS) under the Defense Research & Development Organization (DRDO), Government of India, New Delhi, for their financial support to carry out the research. The authors are grateful to Dr. Atanu Adak and Dr. Tanmay Paul of Vidyasagar University in Paschim Medinipore, India, for their valuable suggestions.

Reference

Anand AC, Sashindran VK and Mohan L. 2006. Gastrointestinal problems at high altitude*. Trop Gastroenterol,* **27:**147–153.

Adak A, Maity C, Ghosh K, Pati BR and Mondal KC. 2013. Dynamics of predominant microbiota in the human gastrointestinal tract and change in luminal enzymes and immunoglobulin profile during high-altitude adaptation. *Folia Microbiol.,***58**:523–528.

Bates JM, Mittge E, Kuhlmn J, Bden Kn, Cheesman SE and Guillemin K. 2006.Distinct signals from the microbiota promote deferent aspects of zebra fish gut differentiation. *Dev Biol.,* **297**:374-386.

Benso A, Broglio F and Aimaretti G. 2007. Endocrine and metabolic responses to extreme altitude and physical exercise in climbers. *Eurp J Endocrinol.,***157**:733-740.

Bhaumik G, Dass D, Bhattacharyya D, Sharma Y and Singh SB. 2013. Heart rate variability changes during first week of acclimatization to 3500 m altitude in Indian Military personnel. *Indian J Physiol Pharmacol.,* **57(1)**:16–22.

Brock FM, Cecil TT, Frosberg W and Buchanan-Smith JG. 1982. Proteolytic activity of rumen microorganisms and effects of proteinase inhibitors. *Appl Environ Microbiol.,***44**: 556-561.

Burtis CA and Ashwood ER. 1999. **Tietz Textbook of Clinical Chemistry,** 3rd Ed. Philadelphia, PA: WB Saunders. pp 809-861.

Gloster TM, Turkenburg JP, Potts JR, Henrissat B and Davies GJ. 2008. Divergence of catalytic mechanism within glycosidase family provides insight into evolution of carbohydrate metabolism by human gut flora. *Chem Biol.,* **20**:1058-1067.

Gombosov L, Lazurova I, Zakuciova M, Curova K, Kmetova M, Petrasova D and Siegfried L. 2011. Genes of intestinal *Escherichia coli* and their relation to the inflammatory activity in patients with ulcerative colitis and Crohn's disease. *Folia Microbiol.,***56** :367-372.

Gouttebargea V and Hellemondtb FV. 2012. Effect of simulated altitude training on blood components and performance in elite speed skaters. *Eurp J Sport and Exer Sci.,***1(4)**:103-107.

Gritsenko VA, Brudastov A, Zhurlov OS and Chertkobv KL. 2000. The properties of *Escherichia* isolated from the bodies of mice in bacterial translocation after immobilization stress. *Zh Mikrobiol Epidemiol Immunobiol,***1**:37-41.

Hackett PH and Roach RC. 2001. High altitude illness. *N Engl J Med*, **345**:107-114.

Hao WL and Lee WL. 2004. Microflora of the gastrointestinal tract methods in molecular biology. *In*: **Public Health Microbiology: Methods and Protocols**. Spencer JFT and de Spencer ALR, (Ed), Humana Press Inc, Totowa, NJ, pp 491-502.

Holy O and Chmelar D. 2012. Oxygen tolerance in anaerobic pathogenic bacteria. *Folia Microbiol.,***57**:443–446.

Kent TH, Fisher LJ and Marr R. 1972. Glucuronidase activity in intestinal contents of rat and man and relationship to bacterial flora. *Proc Soc Exp Biol Med,* **140**:590-594.

Lemos LN, Fulthorpe RR and Roesch LFW. 2012. Low sequencing efforts bias analyses of shared taxa in microbial communities. *Folia Microbiol.,* **57**:409–413.

Ley RE, Peterson DA and Gordon JI. 2012. Ecological and evolutionary forces shaping microbial diversity in the human intestine. *Cell,* **124**:837–848.

Lowry OH, Rosebrough NJ, Farr AL and Randall RJ. 1951. Protein measurement with the foline phenol reagent. *J Biol Chem.,***193**: 265-275.

Mairbaurl H. 2013. Red blood cells in sports: effects of exercise and training on oxygen supply by red blood cells. *Froint Physiol* DOI: 10.3389/fphys.2013.00332.

Maity C, Adak A., Pathak TK, Pati BR and Mondal KC. 2012. Study on the large intestinal cultivable microflora of rat under varied environmental hyperbaric pressures. *J Microbiol Immunol Infect.,***45**: 281-286.

Maity C, Rana S, Pati BR and Mondal KC. 2009. Effect of graded hyperbaric atmospheric pressure on the quantity and composition of faecal flora. *Microb Ecol Health Dis.,* **21**:104–108.

Miller GL. 1959. Use dinitrosalicylic acid reagent for determination of reducing sugar. *Anal Chem.,* **31**:426-428.

Mizuno M, Savard GK, Areskog NH and Saltin B. 2008. Skeletal muscle adaptations to prolonged exposure to extreme altitude: a role of physical activity?. *High Alt Med and Biol.,* **9**:311–17.

Paula de P and Niebauer J. 2012. Effects of high altitude training on exercise capacity: fact or myth. *Sl and Breath*, **16**:233–239.

Rhee SH, Pothoulakis C and Mayer EA. 2009. Principles and clinical implications of the brain gut enteric microbiota axis. *Nat Rev Gastroenterol Hepatol.*, **6**: 306-314.

Rook GAW and Brunet LR. 2005.Microbes, immunoregulation, and the gut.*Gut,***54**:317-320.

Sabbagh M, Rick W and Schneider S. 1988. A kinetic method for the direct determination of creatinine in serum with 3,5-n dinitrobenzoic acid without deproteinization. *J Clin Chem Clin Biochem.,* **26(1)**:15-24.

Shaov XW and Wan CW. 2005. Effects of psychological stress on small intestinal motility and bacteria and mucosa in mice. *World J Gastroenterol.*, **11**:2016-2021.

Wall R, Ross RP, Shanahan F, O'Mahony L, O'Mahony C and Coakley M. 2009. Metabolic activity of the enteric microbiota influences the fatty acid composition of murine and porcine liver and adipose tissues. *Am J Clin Nutr.,* **89**:1393-1401.

Wehr HM and Frank JH. 2004. **Standard Methods for the Examination of Dairy Products**. 17th ed, APHA Inc, Washington DC.

Wickler SJ and Anderson TP. 2000. Hematological changes and athletic performance in horses in response to high altitude (3,800 m). *Am J Physiol Regul Integr Comp Physiol.,***279**:1176–1181.

JJBS
Jordan Journal of Biological Sciences

Volume 11, Number 2,June 2018
ISSN 1995-6673
Pages 147 - 154

Evaluation of Toxicity and Fertility Effects of *Inula viscosa* Aerial Parts Extract in Male Rats

Manal A. Abbas*, Nagham A. Hameed, Ekbal H. Al-Khateeb and Rana M-N. Al Haj

Faculty of Pharmacy and Medical Sciences, Al-Ahliyya Amman University, Amman 19328, Jordan

Received September 26, 2017; Revised October 25, 2017; Accepted October 28, 2017

Abstract

Inula viscosa (Asteraceae) is a herbaceous perennial Mediterranean plant. Despite its very common use in folk medicine, few studies have evaluated its toxicity. This study was conducted to investigate the toxic effects of this plant on male rats especially on the reproductive system. The flavonoid-rich acetone extract of *I. viscosa* was administered intraperitoneally to mature male rats for 60 days. In addition, a one generation fertility study was performed in order to detect the teratogenic effects of this plant. No statistically significant difference was found in the sperms count, sperms morphology, total serum testosterone level, or number and weight of newborns. Also, no gross morphological defects were observed in newborns of treated and control groups. A histological study demonstrated normal spermatogenesis. In addition, a normal architecture of prostate, liver and kidney was observed. However, some morphological alterations were detected in seminal vesicles. Furthermore, liver and kidney tests were normal. In conclusion, this study suggests that the *I. viscosa* extract has no toxic effects in male rats.

Keywords *Inula viscosa,* Toxicity, Male reproductive system, Fertility, Teratogenic effect, Folk medicine

1. Introduction

Herbal drug therapy is a common practice adopted in folk and alternative medicine and has been used in the treatment of various disorders since ancient times (Nabavizadeh *et al*., 2009). According to Ekor (2013), about 80% of individuals from developing countries use traditional medicine for their primary healthcare needs. Jordan is a country rich in flora regarding the number of plant species (Oran, 2014). It was recorded that 20% of the total flora of Jordan is medicinal plants (Oran and Al-Eisawi, 2014) which are used in folk medicine, and can be used in pharmaceutical industry. Despite the deficiency in the evidence-based safety and efficacy of herbal medicine, herbal drug utilization has been increasing in the developing countries, including Jordan (Akour *et al*., 2016). According to a survey conducted by Bardaweel (2014), 92% of males with infertility problems in Jordan resort to herbalists to treat their problems.

Inula viscosa (syn. *Dittrichia viscosa* (L.) Greuter/ Asteraceae) is a herbaceous perennial Mediterranean plant. It is known as Taioon in Arabic (Hudaib *et al*., 2008), and it can found in ruderal environments (along roads) (Parolin *et al*., 2014). In traditional medicine, *I. viscosa* is used externally as muscle relaxant (Hudaib *et al*., 2008), sedative, antiseptic, for wound healing, treating women infertility, antirheumatic, expectorant and for haemorrhoids (Ali-Shtayeh *et al*., 1998). Internally, *I. viscosa* is used as antipyretic, general tonic, antidiabetic, diuretic, antispasmodic, antihelmintic (Ali-Shtayeh *et al*., 1998; Seca *et al.,* 2014), chronic coughing, and tumors (Oran and Al-Eisawi, 2015). Recently the *I. viscosa* plant has drawn the attention because of its pharmacological activities, such as its antioxidant activity (Assi *et al*., 2015), antimicrobial, anti-inflammatory (Assaf *et al*., 2016), antihypertensive and vasodilator effects (Hakkou *et al.,* 2017). According to three studies conducted *in vitro*, *I. viscosa* extracts exhibited cytotoxic effects on cancer cell lines, but their effect on normal cells was not tested (Merghoub *et al*., 2009; Merghoub *et al*., 2016; Messaoudi *et al.,* 2016). Similarly, *I. viscosa* extracts were effective against cancer lines with variable toxicity against the non-cancerous Vero cell line (Talib and Mahasneh, 2011). The common use of this plant in folk medicine necessitates testing its toxicity *in vivo*.

In local folk medicine, a water bath of *I. viscosa* leaves is used to induce abortion (Akour et al., 2016). Scientific research has revealed that *I. viscosa* extracts has anti-implantation and progesterone lowering activities in pregnant rats (Al-Dissi *et al*., 2001). The wide distribution and the very common use of *I. viscosa* in traditional medicine (Oran and Al-Eisawi, 2015) entailed the investigation of the effects of this plant *in vivo*. Also, long-

* Corresponding author. e-mail: manabbas@yahoo.com.

term assessment studies of its toxicity are lacking especially its effects on the male reproductive system. Therefore, the present study was conducted to shed more light on both areas; toxicity and male rat fertility.

2. Materials and Methods

2.1. Plant Collection and Preparation

The *Inula viscosa* aerial parts were collected from Al Subaihi/Jordan during May-June, 2015. The plant was authenticated by a taxonomist, Prof. Dawud Al-Eisawi of the Department of Biological Sciences at the University of Jordan. The aerial parts were dried at room temperature away from direct sunlight, coarsely grinded, and extracted by maceration in acetone since this extract is reported to be rich in flavonoids (Grande *et al*., 1985). The resulting extract was evaporated to dryness under reduced pressure using rotary evaporator. The dried extract was stored at -20 ºC until used.

2.2. Animals

Six week old Wistar rats weighing (180-200gm) were maintained under standard animal room conditions (23 $\pm$ 2 ºC) with 12 hrs light/12 hrs dark cycle. Pelleted food and tap water were available *ad-libitum*. The animals were acclimatized for one week before being used in the experiment. The experimental animals of this research were handled and treated in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

2.3. Determination of the Median Lethal Dose (LD50)

The toxicity of the *I. viscosa* extract was evaluated by the calculation of intraperitoneal (i.p) LD_{50} which determines the dose that kills 50% of animals. The LD_{50} in rats was determined to evaluate the proper treatment dose to be used in this study. For the LD_{50} determination, BALB/c male mice (weight 20-25 g) were obtained from the Animal House of Al-Ahliyya Amman University. The LD_{50} for the *I. viscosa* acetone extract was determined according to the method of Alawi and Jeryes (1982). Mice were divided into eight groups (ten mice each). The doses of the plant extract ranged from 200 mg/kg to 1000 mg/kg, and were given i.p. Animal behavior was carefully observed for two hours, and the number of dead mice was counted in the experimental groups after twenty-four hours.

2.4. Toxicity and Fertility Study

In this part of the study, nine-week male Wistar rats were used. A total of twenty-four rats were randomly divided into three groups (eight rats each): Group I (high dose group) received 82.95 mg/kg (1/10 of the LD_{50} of the acetone extract of *I. viscosa*). Group II (low dose group) rats received 41.475 mg/kg (1/20 of the LD_{50} of the acetone extract). Group III (control group) received the vehicle of the acetone extract (100 µL of Dimethyl sulfoxide (DMSO) per rat daily). Rats received their I.P treatments once a day, and the treatment period lasted for sixty consecutive days in accordance with the WHO protocol (1983).

On the last day of the treatment, the body weight of each rat was recorded. Kidney, liver, epididymis, prostate and seminal vesicles were carefully removed from each animal and weighed quickly using a sensitive 6-digit analytical balance (Shimadzu, Japan). Tissues were fixed in 10% formalin fluid, dehydrated using graded concentrations of alcohol, and embedded in paraffin. The paraffin sections were then cut at five µM thickness using Leica microtome (Germany), stained with hematoxylin-eosin stain to be used later for the histopathological examination. Sections were examined by two histo-pathologists who were blind to the treatments, namely, Dr Iqbal Al-Qubtan of the The Hashemite University-Zarqa, and Dr. Nasrat Babouq of Biolab, Amman, Jordan. The diameter of seminiferous tubules was measured using an MC 170 HD Leica Camera, Switzerland, and the LAS EZ software.

Blood samples were collected from retro-orbital plexus for biochemical analyses, centrifuged at 2500 rpm for ten min, and stored at -20 ºC until used. The total testosterone level was performed at the Specialty Hospital (Amman–Jordan) using Chemiluminescent Microparticle Immunoassay technology (ARCHITECT i 1000 Testosterone *assay*, Abbott). Total protein**,** alkaline phosphatase (ALP), alanine aminotransferase (ALT), aspartate aminotransferase (AST), blood urea nitrogen and serum creatinine were measured using ARCHITECT c 4000 kit, *Abbott.*

For sperm count and morphology, the right cauda epididymis was removed and opened by a surgical blade in 4 ml of Hank's balanced salts solution (HBSS) to exude epididymal contents. Using a haemocytometer, two hundred sperms were counted per specimen. Sperm morphology was examined at 400X magnification and one hundred sperms were counted per specimen. Abnormalities in sperm head, mid-piece or tail were recorded.

Twenty four untreated virgin Wistar female rats weighing (160-180 g) were used for mating the treated males. On the 55th day of the treatment, each male rat was caged separately with one untreated adult female for five days. All males were sacrificed under ether anesthesia at the end of the mating period. Mated females were housed individually after being examined for vaginal sperms. After birth, the number of viable and dead newborns was recorded. The pups were weighed and examined for signs of gross physical deformity.

2.5. Statistical Analysis

All data were expressed as mean± standard error of the mean (SEM). Statistical analysis was performed using GraphPad Prism 7. Organ weights, sperm count, percentage of deformation in sperm morphology, number and weight of newborns, total serum testosterone, total protein**,** ALP, ALT, AST, urea and serum creatinine were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's test. Differences between means were considered statistically significant at $P < 0.05$.

3. Results

3.1. Determination of the Median Lethal Dose (LD50)

The administration of high doses of *I. viscosa* showed convulsions. The LD_{50} of acetone extract was found to be 829.5 mg/kg.

3.2. Toxicity Study

During this study, animals did not show any change in their general behavior, skin changes, including hair loss, defecation, or other abnormal physiological signs. No statistically-significant difference in organ weight was found between *I. viscosa* treated and control groups except for a higher testis weight in the *I. viscosa*-low dose group (Figure 1). Normal histological architecture of testis was observed (Figure 2 A, B). Cross sections of seminiferous tubule of both *I. viscosa* treated and untreated groups, showed discrete layers of germ cells. Spermatogonia were present on the basal lamina and spermatocytes were arranged inward. No retained spermatids, missing germ cell layers or types, multinucleated giant cells, or sloughing of spermatogenic cells into the lumen were observed. Furthermore, the diameter of seminiferous tubules was not statistically different between the control group (89.4±23.3 µm) and the *I. viscosa*-high dose group (93.1±21.1 µm). The epididymis had normal appearance. It did not contain desquamated germ cells or cell debris (Figure 2 C, D). Also, no changes in prostate were observed (Figure 2 E, F). On the other hand, it was noted that the height of mucosal folds in seminal vesicles were shorter in the *I. viscosa*-treated groups (Figure 2 G, H).

Data in Figure 3 also indicates that the administration of acetone extract of *I. viscosa* for sixty days did not alter serum hepatic enzymes activity, serum total protein and urea in rats belonging to both treated groups in comparison with the control group. Creatinine was normal in the group that received a high dose of *I. viscose*, but lower than that of the control group in the low dose group. Furthermore, the histological examination of liver and kidney sections for all the groups involved in the study revealed a lack of any abnormal appearance in these organs (Figure 3 A,B,C,D).

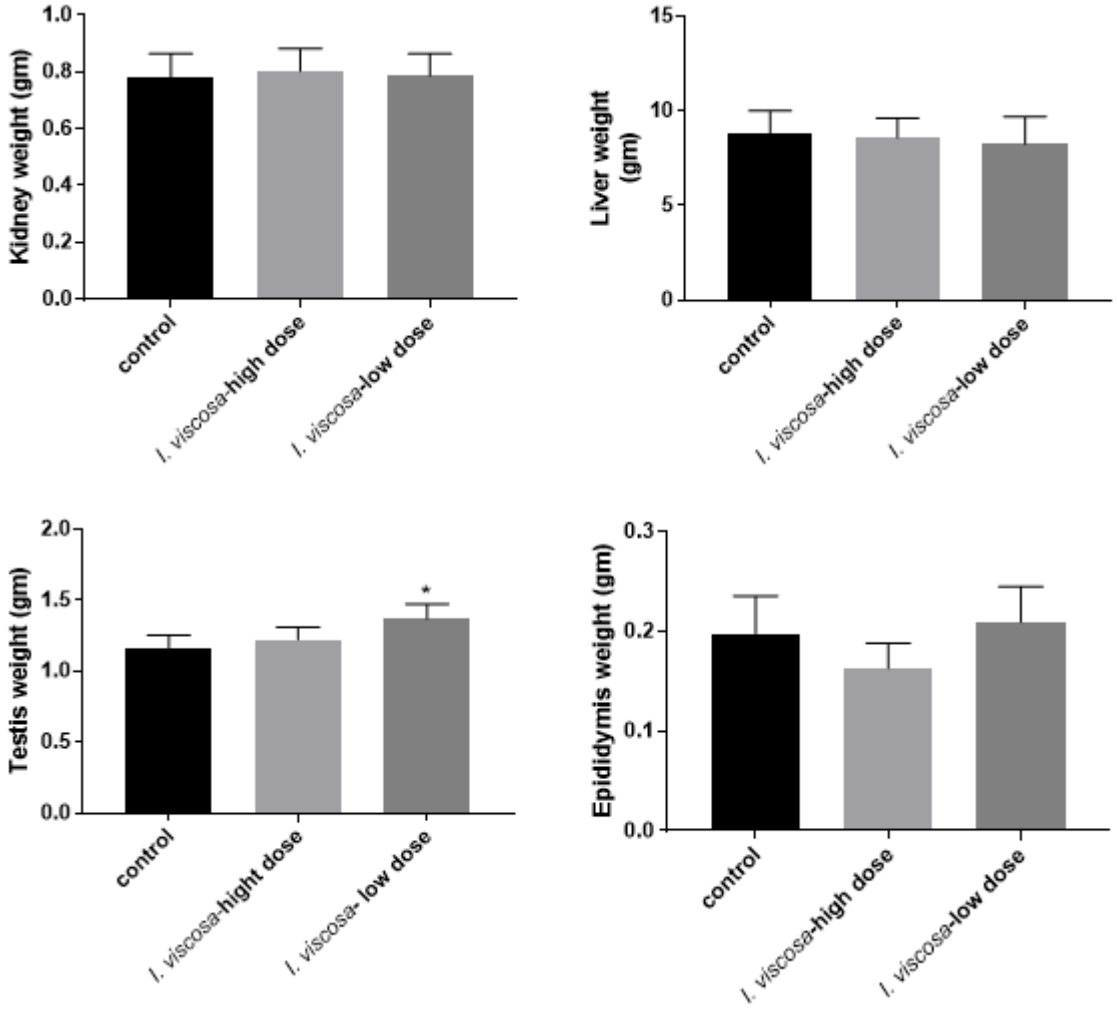


Figure 1. Effect of acetone extract of *I. viscosa* on the organ weight.

* Statistically significant difference at $p<0.05$

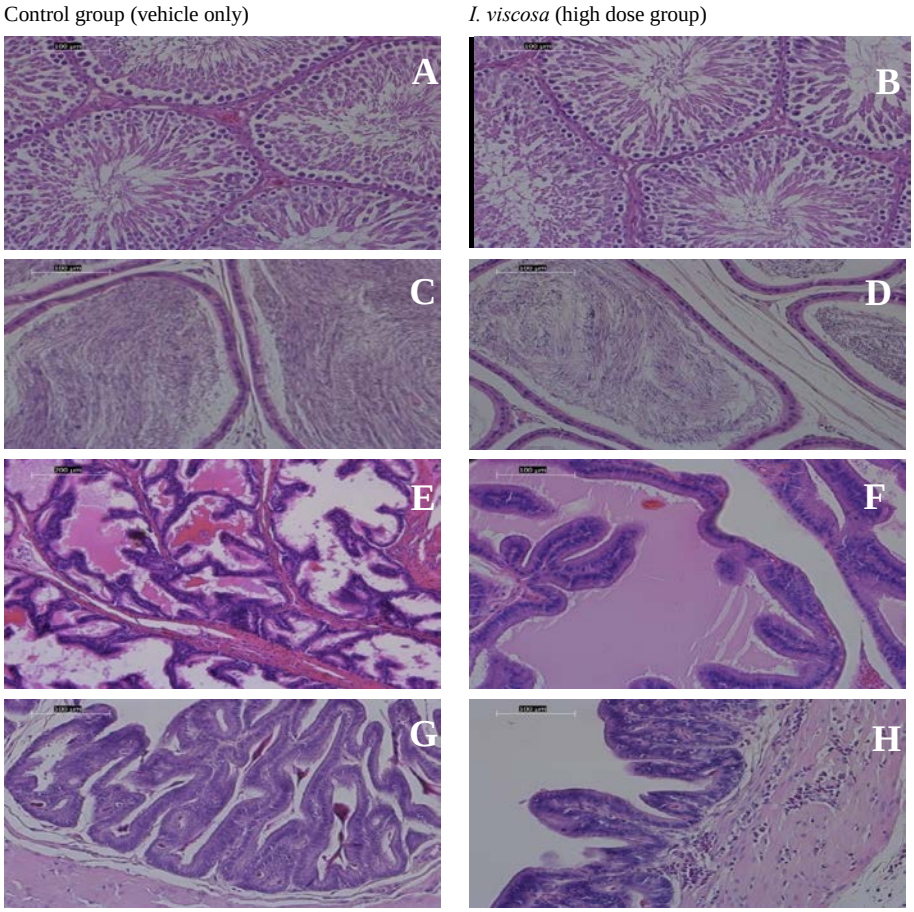


Figure 2. Cross-sections of control and *I. viscosa*-treated groups. (A,B) testis, (C,D) epididymis, (E,F) prostate, and seminal vesicle (G,H). Haematoxylin and eosin stain.

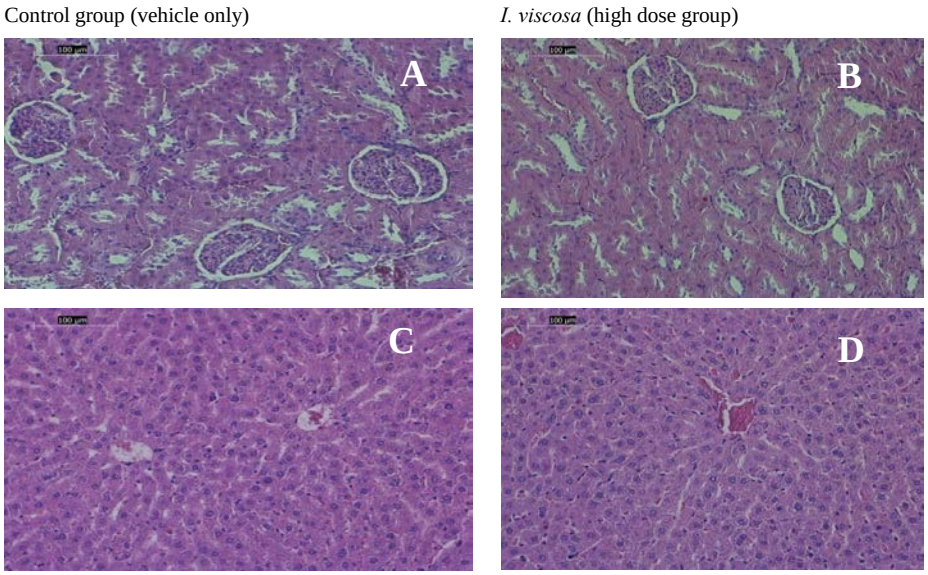


Figure 3. Cross-sections of control and *I. viscosa*-treated groups. (A,B) Kidney, (C,D) liver. Haematoxylin and eosin stain

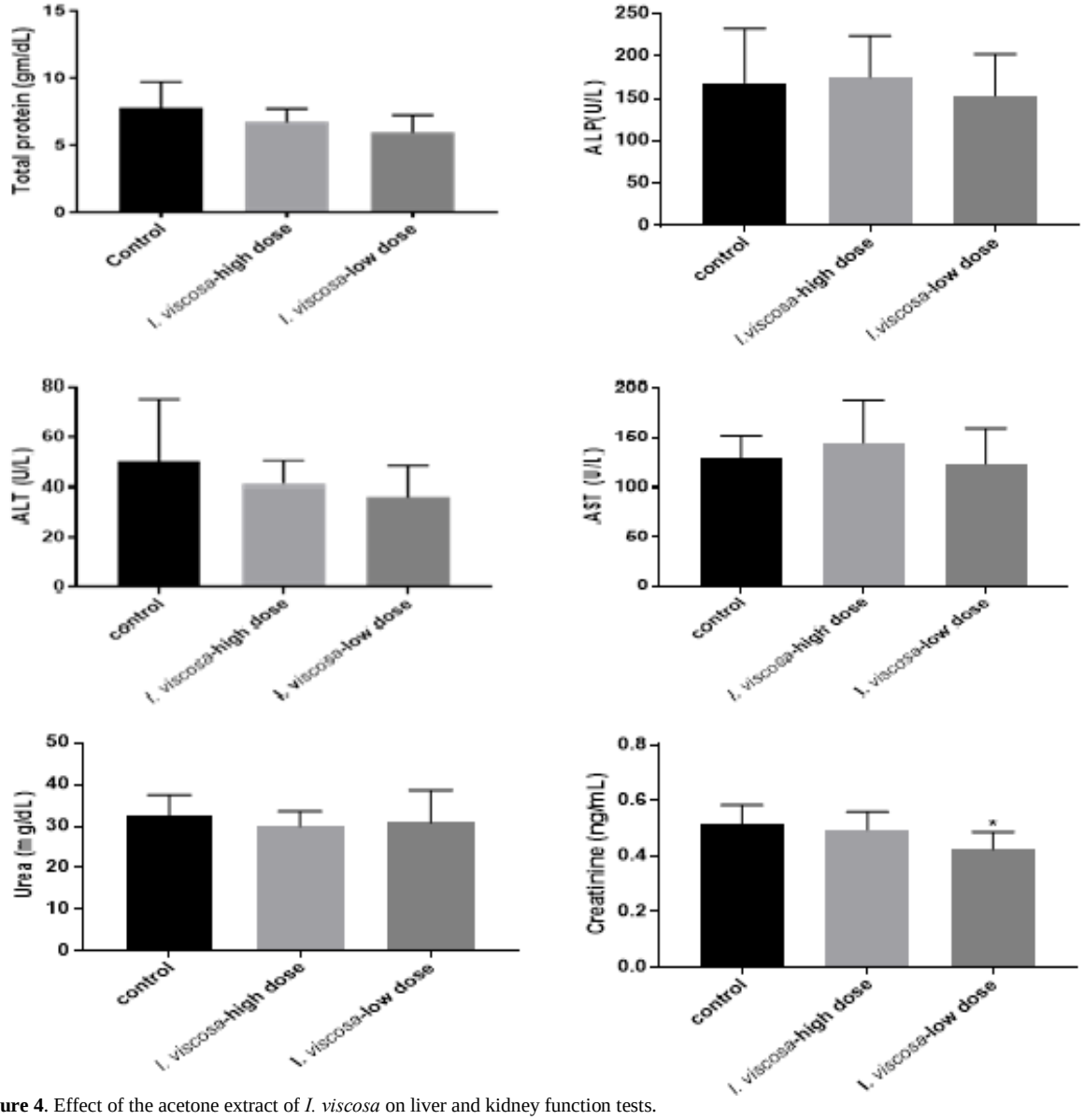


Figure 4. Effect of the acetone extract of *I. viscosa* on liver and kidney function tests.

* Statistically significant difference at $p<0.05$

3.3. Fertility Experiment

The normal sperm count, sperm morphology and the total testosterone level after the administration of various doses of *I. viscosa* acetone extract for sixty days were recorded in comparison with the control group as shown in (Table 1). In the fertility study, the number and weight of newborns in the *I. viscosa* high and low dose-treated groups were not statistically different from those of the control group (Table 1). Furthermore, no gross deformations were observed in newborns.

Table 1. Effect of Acetone extract of *I. viscosa* on sperm count, total testosterone level, number and weight of newborns. Data are represented as mean ± SEM.

Group tested	Sperm count ($x10^6$ sperms/mL)	Sperm abnormalities (% abnormal)	Total testosterone (ng/mL)	Average n° of living newborns	Weight of newborns (gm)
Control group (vehicle)	56.67±5.99	3.02±0.98	3.02±0.98	9.0±0.28	4.96±0.18
High dose acetone extract of *I. viscosa*	52.83±9.80	2.37±0.76	2.37±0.76	11.0±0.45	5.67±0.30
Low dose acetone extract of *I. viscosa*	57.40±2.21	3.23±1.00	1.24±0.34	9.0±1.21	4.96±0.17

* Statistically significant difference at $p<0.05$

4. Discussion

Interest in studying the pharmacological activity of medicinal plants has increased recently. In fact, reports of efficacy are, by far, more numerous than those on toxicity. Therefore, there is an urgent need to study the toxicity of plant extracts (Bello *et al*., 2016; Ghadirkhomi *et al.,* 2016). In this study, no significant differences in kidney, liver and epididymis weights were found upon the administration of the *I. viscosa* aerial part extract. This is an indication that the extract did not alter the size and weight of the organs. As for the control group, the measured values for kidney and liver function tests were found almost normal compared to the normal range published by Charles River laboratories in 2008 (Giknis and Cifford, 2008). Even though, many researchers have already reported factors that affect the serum clinical pathology values in rats such as availability of food before and at the time of bleeding, type of anaesthesia, site of blood sampling, storage conditions, differences in the analytical methods used (Matsuzawa *et al*., 1997), in addition to the environmental conditions, gender, age, origin of breeding system, and feeding (Teixeira *et al*., 2000).

Hepatic enzyme activity was studied to detect any liver malfunction. These enzymatic values went up in acute hepatotoxicity but declined with intoxication due to the damage in the liver (Obi *et al*., 2004). In case of tissue damage, enzymes specific to that tissue are released into circulation. This led to an increase in the activity of these enzymes in the serum (Aliyu *et al*., 2006). Depending on the results clarified in Figure 4, there was no significant difference in the serum ALT, AST, ALP activities between groups receiving different doses of the *I. viscosa* extract and the control group during the treatment period. This indicates that the extract of *I. viscosa* was not harmful to the liver.

The results of the total serum protein and serum urea levels obtained in this study in the control group were within the limits, and are somehow close to the results obtained by other Jordanian researchers (Khouri and Daradka, 2012). The total serum protein may be altered due to liver and/or kidney damage (Fox, 2016). In this experimental study, no significant change in total protein and urea levels was observed between the control and the treated groups. Also, the creatinine level was not raised during the sixty-day treatment with the *I. viscosa* extract. On the contrary, the low dose of the *I. viscosa* extract has significantly lowered the creatinine level. This is an indication that the *I. viscosa* extract was not toxic to the kidney. It is well-known that creatinine is the main catabolic metabolite of the muscle, excreted by the kidneys, and that it is a good indicator of a renal failure (Aliyu *et al*., 2006).

In our sixty-day study, the normal histological morphology of the liver and kidney supports our biochemical results which indicate that the *I. viscosa* extract has no hepatic or renal toxicity. Similar results were obtained by Zaza (2005) who found no change in the liver-enzyme levels (AST, ALT) when aqueous, methanolic and petroleum ether extracts of *I. viscosa* were administered to rats for one month. Atoom (2002) reported that the ethanolic extract of this plant has a slight hepatoprotective effect. In fact, a soft drink of *I. viscosa* is commercially available, and is used to treat non-alcoholic steatohepatitis and other non-alcoholic fatty liver diseases (Assy *et al*., 2012). All these results can be explained by the presence of active constituents in the *I. viscosa* extract, including flavonoids, which are endowed with an antioxidant activity (Assi *et al*., 2015).

An aqueous extract of *I. viscosa* was found to have progesterone lowering effect in pregnant rats (Al-Dissi *et al*., 2001). In our study, no effect of the *I. viscosa* extract on testosterone level was found in male rats. The effect of the *I. viscosa* extract results from the complex interactions between different components that have synergistic and/or antagonistic effects. The acetone extract of *I. viscosa* aerial parts is rich in biologically- active flavonoid aglycones (Grande *et al*., 1985). From this fraction the flavones apigenin, scutellarein 6-methyl ether (hispidulin) and luteolin, 6-methoxyluteolin (nepetin) were isolated (Wollenweber *et al*., 1991). Apigenin was reported to have adverse effects on the male reproductive system in mice (Li *et al*., 2010). On the other hand, luteolin was reported to possess phosphodiesterase type five (PDE5) inhibitory activity (Ko *et al*., 2004) indicating beneficial effects on erection.

Regulatory guidelines for reproductive and fertility toxicity studies consider histopathology as a sensitive and early indicator of spermatogenic disturbances (Creasy, 1997). There are four targets for testicular toxicants. These are: the Sertoli cell, the Leydig cell, the germ cells (each has its own specific chemical sensitivity) and the vascular endothelium. In this study, no change in the histological architecture of the testis and epididymis was found. Also, there was no change in the seminiferous tubule diameter. In addition, the number of epididymal sperms was comparable to that of the control group. Furthermore, no change in testosterone level was found between the control group and the *I. viscosa* treated groups. Therefore, it can be concluded that the *I. viscosa* extract is unlikely to have a harmful effect on testis. A higher testicular weight was found in the *I. viscosa*-low-dose group compared to the control group while no change in epididymis weight was observed between the control and the *I. viscosa*-treated groups (Figure 1). According to Creasy (1997) if a significant decrease in testis weight was found, it is expected to find an obvious germ cell loss. On the other hand, if there is no significant reduction in testis and epididymis weights, it is unlikely to have a clear change in testicular morphology.

Since accessory sex glands are androgen-dependent, they may reflect changes in the endocrine status and testicular function (Campion *et al*., 2012). Seminal vesicle mucosal fold height was lower in the *I. viscosa* treated groups. Further research is needed to investigate this aspect. To our knowledge, the results of this study are the first to demonstrate the effect of the *I. viscosa* acetone extract on male rat fertility.

5. Conclusion

In relation to the experiments performed in this study, one can conclude that the administration of *I. viscosa* acetone extract for sixty days at the doses investigated had no toxic effect on male fertility and organ toxicity in rats.

This may support the safety of using this plant in Jordanian folk medicine. However, further studies are needed either to ascertain its safety or to investigate the effects of its active constituents and pure compounds (after isolation) in humans.

Acknowledgment

The authors thank Prof. Dawud Al-Eisawi (Department of Biological Sciences/The University of Jordan) for plant authentication. Also, we would like to thank Dr Iqbal Al-Qubtan; a pathologist at The Hashemite University/Jordan for the histopathological examination. This project was funded by the Deanship of Graduate Studies and Scientific Research/Al-Ahliyya Amman University, Jordan (Grant number 5/7/2013-2014).

Conflict of interest

The authors declare no conflict of interest.

References

Akour A, Kasabri V, Afifi FU and Bulatova N. 2016. The use of medicinal herbs in gynecological and pregnancy-related disorders by Jordanian women: a review of folkloric practice vs. evidence-based pharmacology. *Pharm Biol*, **54**(9):1901-18.

Alawi A and Jeryes A. 1982. The lethal dose of the Jordanian scorpion *Leiurus quinquestriatus* venom. *Iraqi J Sci*, **23**:459–469.

Al-Dissi NM, Salhab AS and Al-Hajj H. 2001. Effects of *Inula viscosa* extracts on abortion and implantation in rats. *J Ethnopharmacol*, **77**:117–121.

Ali-Shtayeh MS, Yaghmour R M-R, Faidi YR, Salem K and Al-Nuri MA. 1998. Antimicrobial activity of 20 plants used in folkloric medicine in the Palestinian area. *J Ethnopharmacol*, **60**:265–271.

Aliyu R, Adebayo AH, Gatsing D and Garba IH. 2006. The effects of ethanolic extract of *Commiphora africana* (burseraceae) on rat liver and kidney functions. *J Pharmacol Toxicol*, **2**: 373-379.

Assaf AM, Amro BI, Mashallah S and Haddadin RN. 2016. Antimicrobial and anti-inflammatory potential therapy for opportunistic microorganisms. *J Infect Dev Ctries*, **10**(5):494-505.

Assi M, Ela MA, Raafat K and El-Lakany A. 2015. Role of antioxidants in the antidiabetic potential of two indigenous Lebanese *Inula* species. *Med Aromat Plants*, **S2**: 002.

Assy N, Grozovski M and Maoz M. A cola beverage containing *Inula viscosa* extract for consumers suffering non-alcoholic fatty liver disease (NAFLD). Patent WO 2012107918 A1, EP, 2012.

Atoom MM. 2002. Some pharmacological studies of ethanolic extract of *I. viscosa* leaves. Msc thesis, Jordan University of Science and Technology, Irbid, Jordan.

Bardaweel, SK. 2014. Alternative and antioxidant therapies used by a sample of infertile males in Jordan, a cross-sectional survey. *BMC Complement Altern Med*, **14**:244.

Bello I, Bakkouri A, Tabana Y, Al-Hindi B, Al-Mansoub M, Mahmud R and Asmawi M Z. 2016. Acute and sub-acute toxicity evaluation of the methanolic extract of *Alstonia scholari*s stem bark. *Med Sci*, **4**(1):4.

Campion S, Catlin N, Heger N, McDonnell EV, Pacheco SE. Saffarini C, Sandrof MA and Boekelheide K. 2012. Male reproductive toxicity and endocrine disruption. *EXS*, **101**:315–360.

Creasy DM. 1997. Evaluation of testicular toxicity in safety evaluation studies: The appropriate use of spermatogenic staging. *Toxicol Pathol*, **25**(2):119-131.

Ekor M. 2013. The growing use of herbal medicines: issues relating to adverse reactions and challenges in monitoring safety. *Front Pharmacol*, **4**:177.

Fox SI. 2016. **Human Physiology**, 14th ed., McGraw-Hill Companies, pp. 458.

Ghadirkhomi A, Safaeian L, Zolfaghari B, Aghaye-Ghazvini MR and Rezaei P. 2016. Evaluation of acute and sub-acute toxicity of *Pinus eldarica* bark extract in Wistar rats. *Avicenna J Phytomed,* **6**(5):558-566.

Giknis MLA and Cifford CB. 2008. **Clinical Laboratory Parameters for CRI**: WI (Han), Charles River Laboratories, pp8.

Grande M, Piera F, Cuenca A, Torres P and Bellido IS. 1985. Flavonoids from *Inula viscosa. Planta Med*, **5**:414-419.

Hakkoua Z, Maciuk A, Leblais V, Bouanania NE, Mekhfia H, Bnouhama M, Aziza M, Ziyyata A, Rauf A, Ben Hadda T, Shaheenf U, Patelg S, Fischmeister R and Legssyer A. 2017. Antihypertensive and vasodilator effects of methanolic extract of *Inula viscosa*: Biological evaluation and POM analysis of cynarin, chlorogenic acid as potential hypertensive. *Biomed Pharmacother*, **93**:62–69.

Hudaib M, Mohammad M, Bustanji Y, Tayyema R, Yousef M, Abuirjeie M and Aburjai T. 2008. Ethnopharmacological survey of medicinal plants in Jordan, Mujib Nature Reserve and surrounding area. *J Ethnopharmacol*, **120**:63–71.

Khouri NA and Daradka H. 2012. Blood biochemical changes associated with acute treatment of *Orchis anatolica* plant roots ethanol extract in adult albino rats. *IJABPT*, **3**(1):73-81.

Ko WC, Shih CM, Lai YH, Chen JH and Huang HL. 2004. Inhibitory effects of flavonoids on phosphodiesterase isozymes from guinea pig and their structure-activity relationships. *Biochem Pharmacol*, **68**(10):2087-2094.

Li H, Li H-B, Zhang M, Yan F, Zhang Z-X and Li Z-L. 2010. Effect of apigenin on the reproductive system in male mice. *Health*, **2**(5):435-440.

Matsuzawa T, Hayashi Y, Nomura M, UnnoT, Igarashi T , Fyruya T , Sekita K, Ono A, Kurokawa Y and Hayashi Y. 1997. A survey of the values of clinical chemistry parameters obtained for a common rat blood sample in ninety-eight Japanese laboratories. *J Toxicol Sci*, **22**(1):25-45.

Merghoub N, Benbacer L, Amzazi S, Morjani H and El Mzibri M. 2009. Cytotoxic effect of some Moroccan medicinal plant extracts on human cervical cell lines. *J Med Plants Res*, **3**:1045-1050.

Merghoub N, El Btaouri H, Benbacer L, Gmouh S, Trentesaux C, Brassart B, Terryn C, Attaleb M, Madoulet C, Benjouad A, Amzazi S, El Mzibri M and Morjani H . 2016. *Inula viscosa* extracts induces telomere shortening and apoptosis in cancer cells and overcome drug resistance. *Nutr Cancer*, **68**(1):131–143.

Messaoudi M, Chahmi N, El Mzibri M, Gmouh S, Amzazi S, Benbacer L and El Hassouni M. 2016. Cytotoxic effect and chemical composition of Inula viscosa from three different regions of Morocco. *Europ J Med Plants* **16**(4):1-9.

Nabavizadeh SH, Majid O-b and Anushiravani A. 2009. Direct *ex vivo* effects of herbal extracts on serum bilirubin in neonatal blood samples. *AJBR*, **3**(5):226-228.

Obi E, Orisakwe OE, Asomugha, LA and Udemezue, OO. 2004. The hepatotoxic effect of halofantrine in guinea pigs. *Indian J Pharm Sci*, **36**(5):303-305.

Oran SA. 2014. The status of medicinal plants in Jordan. *JAST*, **A4**:461-467.

Oran SAS and Al-Eisawi DMH. 2015. Ethnobotanical survey of the medicinal plants in the central mountains (North-South) in Jordan. *J Bio Env Sci*, **6**(3):381-400.

Oran SA and Al-Eisawi D. 2014. Medicinal plants in the high mountains. *Int J Biodivers conserv*, **6**(6):436-443.

Parolin P, Scotta MI and Bresch C. 2014. Biology of *Dittrichia viscosa*, a Mediterranean ruderal plant: a review. *Int J Exp Bot*, **83**:251-262.

Seca AM, Grigore A, Pinto DC and Silva AM. 2014. The genus *Inula* and their metabolites: From ethnopharmacological to medicinal uses. *J Ethnopharmacol*, **154**(2):286-310.

Talib WH and Mahasneh AM. 2011. Antiproliferative activity of plant extracts used against cancer in traditional medicine. *Sci Pharm*, **78**:33–45.

Teixeira MA, Chaguri LCAG, Carissimi AS, De Souza NL, Mori CM C, Gomes VMW, Neto AP, Nonoyama K and Merusse JLB. 2000. Hematological and biochemical profiles of rats (*Rattus norvegicus*) kept under microenvironmental ventilation system. *Braz J Vet Res Anim Sci*, **37**(5):341-347.

WHO. Protocol MB-50: A method for examining the effect of the plant extracts administered orally on the fertility of male rats (APF/IP, 9914E). World Health Organization 1983, Geneva.

Wollenweber E, Mayer K and Roitman JN. 1991. Exudate flavonoids of *Inula viscosa*. *Phytochem.,* **30**(7):2445–2446.

Zaza RM. 2005. The effect of *Inula viscosa* extract on inflammation, microbial growth, glycemia, and blood lipid profile. MSc thesis, Lebanese American University, Lebanon.

JJBS

Volume 11, Number 2,June 2018
ISSN 1995-6673
Pages 155 - 162

Jordan Journal of Biological Sciences

The Impact of the 7-Valent Pneumococcal Conjugate Vaccine on Nasopharyngeal Carriage of *Streptococcus pneumoniae* in Infants of Ajlun Governorate in Jordan

Adnan Al-Lahham[1,*], Jumana Abu Qayyas[1] and Mark van der Linden[2]

[1]*School of Applied Medical Sciences, German Jordanian University, P.O. Box 35247, 11180 Amman, Jordan;*

[2]*Institute of Medical Microbiology, National Reference Center for Streptococci, University Hospital of Aachen, Germany*

Received September 13, 2017; Revised October 31, 2017; Accepted November 4, 2017

This paper was presented in part at the 26th International Pediatric Association Congress, Johannesburg, South Africa 3-9 August 2010. Poster 917; and at the 29th ESPID conference, in The Hague- Netherlands from 7-11 June 2011 with poster number 595. It was registered in the website: ClinicalTrials.gov under the identifier number NCT00900978 and was funded in part by Pfizer and the German Jordanian University.

Abstract

Streptococcus pneumoniae colonization is a serious problem since dissemination to other organs in the body can cause serious infections. This study investigates the impact of the pneumococcal conjugate vaccine (PCV7) on pneumococcal carriage and resistance in healthy infants in the rural areas of Ajlun, Jordan. 415 infants younger than two years of age were selected for this study. The vaccination was given at ages 2, 4 and 10 months between April 2009 and July 2010 (Scheme 2+1). Nasopharyngeal (NP) swabs were taken at the first vaccination (phase one), the third vaccination (phase two), and also three months after the last vaccination (phase three). Pneumococcal isolates were identified according to morphology, bile solubility, and optochin sensitivity. Antimicrobial susceptibility was tested via the micro-broth dilution method and serotyping by the (Neufeld) Quellung reaction. At the first vaccination, carriage in two-month old infants was 26.3%. At the third vaccination, carriage in ten-month–old infants was 29.9%, and at three months after their last vaccinations it was 29.4%. Twenty cases were found positive for all the three nasopharyngeal swabs (5.1%). At the end of the study 241/415 cases (58.1%) were carriers. Resistance rates were as follows: (intermediate and resistant): penicillin (84.0%), cefotaxime (5.6%), clarithromycin (47.2%), clindamycin (25.6%), trimethoprim-sulfamethoxazole (59%), and tetracycline (39%). Multidrug resistance rate was 39.5% for phase one, 48.4% for phase two, and 46.7% for phase three. Frequent serotypes at the time of the first vaccination were: 6A (14.7%), 19F (12.8%), 6B, 23F, and 15B at 6.4% each, and 11A (5.5%). Frequent serotypes after the last vaccination were: 11A (12.3%), 19A (10.7%), 6A (9.0%), and 19F (8.2%). The prevalence of vaccine serotypes (4, 6B, 9V, 14, 19F and 23F) at phase one (0.9%, 6.4%, 1.8%, 3.7%, 12.8%, 6.4%, respectively) was reduced at phase three (0.0%, 2.5%, 0.0%, 3.3%, 8.2%, 0.8%, respectively). Serotype 18C was not identified. An increase of 19A in phase one from 2.8% to 10.7% at phase three was observed. The impact of vaccination was an observed reduction in the resistance to penicillin, cefotaxime, clarithromycin, and clindamycin by 5.9%, 2.4%, 11.4%, and 18.3%, respectively. Coverage of the PCV7 and PCV13 three months after receiving the third injection was 27.8% and 49.4% respectively. High carriage and resistance rates were observed among the infants. Vaccine administration reduced pneumococcal carriage and antimicrobial resistance among the infants.

Keywords: *Streptococcus pneumoniae*, Carriage, Resistance, Pneumococcal Conjugate Vaccines.

1. Introduction

Streptococcus pneumoniae is an infectious agent causing meningitis, pneumonia, and bacteraemia especially for young children, mainly in low-income countries where pneumococcal conjugate vaccines (PCVs) are still underused. Understanding the epidemiology of carriage for *Streptococcus pneumoniae* (*S. pneumoniae*) and other common respiratory bacteria in developing countries is crucial for implementing appropriate vaccination strategies and evaluating their impact (Adegbola *et al*., 2014). Nasopharyngeal colonization with *S. pneumoniae* in infants is generally acquired at approximately 4-6 months of age (Al-Lahham and Van der Linden 2014; Bogaert *et al*., 2004), and is considered a prerequisite for a disease. Unlike children, carriage in the elderly is rarely detected (Krone *et al*., 2015). Pneumococcal disease will not occur without preceding nasopharyngeal colonization with the homologous strain. Therefore, pneumococcal carriage is believed to be an

* Corresponding author. e-mail: adnan.lahham@gju.edu.jo.

important source of a horizontal spread of this pathogen within the community (Bogaert *et al*., 2004). Increased prevalence of *S. pneumoniae* in the nasopharynx of healthy paediatrics reflects a potential risk to the development of more frequent respiratory infections in the community (Saha *et al*., 2003; Volonakis *et al*., 2006). *S. pneumoniae* was given the name of the forgotten killer in children by the WHO (Wardlow, 2006). As stated by the WHO in 2007 at least 1.2 million children die of pneumococcal infections each year, with 70% of them being from Africa and southeast Asia; mostly from developing countries (Williams, 2002). It accounts for more than one third of acute bacterial sinusitis and more than one half of community-acquired bacterial pneumonia (File, 2006). Resistance of *S. pneumoniae* to antimicrobials makes the treatment more difficult. On the other hand, the emergence of penicillin- and cephalosporin-resistant strains has created an urgent need for pneumococcal vaccines that are effective in treating infants (Jacobs and Dagan, 2004; Pallares *et al*., 2003). Pneumococcal vaccines containing capsular polysaccharides of five (6B, 14, 19F, 18C, 23F) and seven (4, 6B, 9V, 14, 19F, 18C, 23F) serotypes have been proven safe and immunogenic in children (Wardlow, 2006).

Pneumococcal infections are particularly common in younger children and in older adults. They can be divided broadly into invasive and non-invasive diseases (Ludwig, 2013; Ludwig *et al*., 2012). Pneumonia is one of the most common clinical presentations of pneumococcal infections, and may itself be invasive or non-invasive (Amodio *et al*., 2014; Said *et al*., 2013).

Although there are differences in the prevalence and rank order of the serotypes obtained from NP specimens and from those with invasive diseases, the pneumococcal nasopharyngeal isolates may reflect the strains circulating in the community. They can be used as a marker to predict the serotype prevalence of invasive diseases and resistance patterns. Monitoring serotype distribution is essential for the appropriate application of the vaccines. Vaccine use in infants has proved highly efficacious in the prevention of the Invasive Pneumococcal Disease as well as in decreasing the carriage of vaccine serotypes in the nasopharynx of infants, which significantly affects, in the long run, the occurrence of otitis media and helps decrease infection rates among contacts of the vaccinated infants. Information concerning the pneumococcal strains found in Jordanian children and the NP carriage in infants is limited, and does not include children living in rural areas. A population-based study was undertaken to determine the impact of the vaccination of healthy infants with the PCV7 in Ajlun, the first district in Jordan where vaccine (PCV7) was used. The aims of this study were to determine the frequency of *S. pneumoniae* NP carriage and serotypes circulating among infants aged 2-18 months in the rural areas of Ajlun, to identify the antimicrobial susceptibility of these isolates, and to study the impact of the pneumococcal conjugate vaccine on carriage and resistance.

2. Material and Methods

2.1. Study Design

A 15-month population based surveillance study of pneumococcal NP carriage and the antimicrobial resistance of *S. pneumoniae* in healthy Jordanian infants was conducted in Ajlun on babies born between March and April of 2009. The first NP-swab was taken by a medical doctor at the time of the first vaccination at 2-3 months of age (phase one). The second NP-swab was taken at the time of the second vaccination at ages 4-5 months (phase two). The third vaccination was at age of 10-11 months (phase three). The third NP-swab was taken three months after the last vaccination, i.e. at age of 13-14 months. Infants were included in the study after obtaining a signed parental consent. Vaccination was free of charge and took place at the 12 main Day Care Centers (DCCs) in the governorate of Ajlun. PCV7 and PCV10 are pneumococcal conjugate vaccines available for use only in the private sector. PCV7 was the only administrated pneumococcal vaccine for this study. Doses were donated by Wyeth Pharmaceutical Company (now called Pfizer) to the Ministry of Health in Jordon in 2008. All the 415 infants, included in the study, received the three vaccine doses. Special questionnaires and forms were completed for each sample. Swabs were analysed at a reference laboratory of the German Jordanian University and at the National Reference Center for Streptococci in Aachen, Germany. The protocol was approved by the Ministry of Health (MOH) and the ethical committee of the MOH, before the study was undertaken.

2.2. Culture and Identification

The NP samples were inoculated on Columbia Agar plates with 5% sheep blood. The plates were incubated overnight at 35°C in 5% CO_2. Identification was performed by conventional microbiological methods; colony morphology, susceptibility to optochin (bioMérieux), and bile solubility.

2.3. Susceptibility Testing

Minimal inhibitory concentration (MIC) testing was performed using the micro broth dilution method as recommended by the Clinical Laboratory Standards Institute (CLSI) (CLSI, 2017). Antimicrobial agents used were penicillin G, amoxicillin, cefotaxime, cefuroxime, cefpodoxime, clarithromycin, clindamycin, tetracycline, levofloxacin, moxifloxacin, telithromycin, trimethoprim/ sulfamethoxazole, chloramphenicol, and vancomycin. *S. pneumoniae* ATCC 49619 was used as a control strain. Multidrug resistance phenotype was recorded when an isolate had resistance to three or more classes of antimicrobial agents.

2.4. Analysis of Resistance Determinants (genotyping and phenotyping)

PCR of macrolide resistance determinants was performed as described previously by (Reinert *et al*., 2004; Reinert *et al*., 2005; Reinert *et al*., 2003). For the classical detection of *erm*(B) and *mef* (A) the following primers were used: *erm*(B) 5´-CGAGTGAAAA AGTACTCAACC-3´ (362-382) and 5´-GGCGTGTTT CATTGCTTGATG-3´ (978-958), *mef* (A) 5´-AGTAT CATTAATCACTAGTGC-3´ (57-77) and 5´-TAATAG ATGCAATCACAGC-3` (550-532). Results were confirmed by a Light Cycler protocol with the following primers: *erm*(B) 5´-TTTTGAAAGCCATGCGTCTGA-3´, and 5´-ATCTGTGGTATG GCGGGTAAGTT-3´, and *mef*(A) 5´-TATGGGCAGGGCAAGCAGTATC-3´ and 5´-TCRGCACCAATCATTATCTTCTTC-3´ (Farrell *et*

al., 2001). The macrolide resistance phenotype was determined on the basis of the pattern of susceptibility to MLS_B (macrolide-lincosamide-streptogramin B) (Montanari *et al*., 2001).

2.5. Serotyping

Capsular typing of the pneumococcal isolates was performed by the Neufeld's Quellung reaction using type and factor sera provided by the Statens Serum Institute, Copenhagen, Denmark.

2.6. Primary and Secondary Endpoints

Endpoints included the frequency of NP carriage, serotype distribution, and antimicrobial resistance patterns of the strains in infants younger than two years old in rural areas. They were attending DCCs for normal check-ups and for vaccination through the National Immunization Program. Assessment of the reduction in vaccine-type pneumococcal carriage as a result of vaccination was determined in order to model a routine schedule of vaccination with PCV7.

3. Results

Of the 415 infants included in the study, the overall carriage rate at the end of the study was 58.1% (i.e., 241 infants were carriers at any phase of the study). 171 male infants had a carriage rate of 55.3%, and 244 females had a carriage rate of 62.0%. A total of twelve DCCs were included in the study, each having 5-110 infant participants (Table 1). At phase one, 26.3% of the infants were tested positive, at phase two, 29.6% were found positive, and at phase three 29.4% of the cases were pneumococci positive. Among the 12 centers, the highest carriage rate (70%) was recorded in Halawa at phase one, which was reduced at phase two to 50%, and then to 40% at phase three. Rajeb center had the highest rate of carriage (80%) at phase three. The resistance ranges in the three phases were: penicillin G (82%-82.5%); cefotaxime (4.9%-7.3%); clarithromycin (40.4%-55.7%); clindamycin (18.0%-36.3%); tetracycline (33.6%-46.8%); and trimethoprim-sulfamethoxazole (56%-62.3%). No resistance was shown for vancomycin, levofloxacin, moxifloxacin, and telithromycin (Table 2). The most commonly detected serotypes in phase one were 6A (14.7%), 19F (12.8%), 6B (6.4%), 15B (6.4%), 23F (6.4%), 11A (5.5%) and 24F (5.5%); at phase two: 19F (18.6%), 6A (12.9%), 19A (8.1%), 6B (8.1%), 11A (5.7%) and 35B (4.8%), and at phase three: 11A (12.3%), 19A (10.7%), 6A (9.0%), 19F (8.2%), 15B (6.6%), 15A (4.9%), 23A (4.9%) and 35B (4.9%) (Table 3). Serotypes 1, 5 and 18C were not recovered during the study. Vaccine coverage (PCV7, PCV10 and PCV13) for the three phases was as follows: phase one (32.1%, 32.1%, 50.5%), phase two (33.9%, 33.9%, 48.4%) and phase three (14.8%, 15.6%, 36.9%). The carriage rate was 26.3% at phase one, 29.6% at phase two, and 29.4% at phase three. However, there was a reduction in the prevalence of PCV7 serotypes from 32.1% at phase one to 14.8% at phase three, and for PCV13 from 50.5% at phase one to 36.9% at phase three (Table 2). An increase of the 19A from 2.8% in phase one to 10.7% at phase 3 was noticed (Table 3). The effectiveness of the vaccination was also observed in the reduction of the isolate's resistance to penicillin, cefotaxime, clarithromycin, and clindamycin by 6%, 2.4%, 11.4%, and 17.7%, respectively (Table 2). Table 4 shows the carriage rate at each phase with vaccine and non-vaccine serotypes, and whether carriers showed positive *S. pneumoniae* more than one time during the study. On the whole, twenty infants (5.1%) were positive for the three NP swabs taken during the study period. For all DCCs, the rate of non-vaccine serotypes isolated from infants at phase one was 49.5% and 63.1% at phase three (Table 5). Macrolide resistance for all study isolates was 49%, where $cMLS_B$ phenotype was the most prevalent at 50.8%, and *erm*B was most prevalent genotype at 50% (Table 6). There were 75 infants carrying *S. pneumoniae* PCV7 serotypes at the end of the second vaccination from all the twelve DCCs, while 76% of these infants showed no PCV7 serotypes at the end of the study (Table 7). Among these 75 infants, the number of carrier infants with possible serotype shifts from 19F to 19A, and from 6B to 6A at the end of the study was seven (9.3%) (Table 7). Multidrug resistance (MDR) rates among the pneumococcal isolate were 39.5% at phase one, 48.4% at phase two, and 46.7% at phase three (Table 8).

Table 1. Distribution of participants by center in Ajlun.

Day Care Center	Number of recruited infants
Ajlun City	35
Alhashmia	08
Anjara	97
Arjan	22
Baaoon	09
Ebben	35
Ein Janna	22
Eshtafena	11
Halawa	10
Kafranjeh	110
Rajeb	05
Sakhra	51
Total	415

Table 2. Antimicrobial resistance and coverage of pneumococcal conjugate vaccines for the three phases.

	Resistance rate % (intermediate and resistant)						Vaccine serotypes (%)		
Phase	Pen	Ceta	Cla	Cli	Tet	Sxt	PCV7	PCV10	PCV13
I (n=109)	82.5	7.3	40.4	22.0	35.8	56.0	32.1	32.1	50.5
II (n=124)	87.9	4.8	55.7	36.3	46.8	58.1	33.9	33.9	55.7
III (n=122)	82.0	4.9	44.3	18.0	33.6	62.3	14.8	15.6	36.9
*Total	84.0	5.6	47.2	25.6	39.0	59.0	27.0	27.3	47.8

Abbreviations: Pen, penicillin G; Ceta, cefotaxime; Cla, clarithromycin; Cli, clindamycin; Tet, tetracycline; Sxt, trimethoprim/sulfamethoxazole. Breakpoints (I, R) according to CLSI 2017: penicillin G; 0.1–1 µg/mL, ≥2 µg/mL; cefotaxime; (non-meningitis): 2 µg/mL, ≥4 µg/mL; clarithromycin; 0.5 µg/mL, ≥1 µg/mL; clindamycin: 0.5 µg/mL, ≥1 µg/mL; trimethoprim/ sulfamethoxazole; 1/19-2/38 µg/mL, ≥4/76 µg/mL.

*Total = 356 isolates were isolated from all recruited infants (415). Each of the 415 infants in Ajlun governorate provided 3 nasopharyngeal swabs during the study. Some infants were positive in one swab others were positive 2 or 3 times (Table 4).

Table 3. Number and rate (%) of serotypes detected at each phase.

	Phase I		Phase II		Phase III	
Serotype	Nr.	%	Nr.	%	Nr.	%
6A	16	14.7	16	12.9	11	9.0
19F	14	12.8	23	18.6	10	8.2
6B	7	6.4	10	8.1	3	2.5
15B	7	6.4	5	4.0	8	6.6
23F	7	6.4	4	3.2	1	0.8
11A	6	5.5	7	5.7	15	12.3
24F	6	5.5	2	1.6	1	0.8
14	4	3.7	4	3.2	4	3.3
15A	4	3.7	3	2.4	6	4.9
23A	4	3.7	4	3.2	6	4.9
16F	4	3.7	4	3.2	4	3.3
35B	4	3.7	6	4.8	6	4.9
NT	4	3.7	2	1.6	4	3.3
15C	3	2.8	2	1.6	2	1.6
17F	3	2.8	2	1.6	2	1.6
19A	3	2.8	10	8.1	13	10.7
10A	2	1.8	1	0.8	2	1.6
33F	2	1.8	4	3.2	3	2.5
9V	2	1.8	1	0.8	0	0
3	1	0.9	1	0.8	2	1.6
21	1	0.9	1	0.8	0	0
42	1	0.9	1	0.8	0	0
7C	1	0.9	1	0.8	0	0
9N	1	0.9	2	1.6	5	4.1
4	1	0.9	0	0	0	0
28A	1	0.9	0	0	0	0
16B	0	0	1	0.8	0	0
35C	0	0	1	0.8	0	0
35F	0	0	1	0.8	1	0.8
33A	0	0	2	1.6	1	0.8
13	0	0	1	0.8	3	2.5
34	0	0	1	0.8	2	1.6
10F	0	0	1	0.8	2	1.6
7B	0	0	0	0	2	1.6
18A	0	0	0	0	1	0.8
7F	0	0	0	0	1	0.8
31	0	0	0	0	1	0.8
Total	109	100%	124	100%	122	100%

Nr., number

Table 4. Carriage rate of *S. pneumoniae* with vaccine and non-vaccine serotypes.

	Carriage rate	Vaccine serotypes, n (%)			Non-vaccine serotypes
	n (%)	PCV7	PCV10	PCV13	n (%)
Phase I (n=415)	109 (26.3)	35 (32.1)	35 (32.1)	55 (50.4)	54 (49.6)
Phase II (n=415)	124 (29.9)	42 (33.9)	42 (33.9)	60 (55.7)	64 (51.6)
Phase III (n=415)	122 (29.4)	18 (14.8)	19 (15.6)	45 (36.9)	77 (63.1)
[1]Positive only in Phase I	49 (11.8)	14 (28.6)	14 (28.6)	25 (51)	24 (49)
[2]Positive only in Phase II	42 (10.1)	14 (33.3)	14 (33.3)	22 (52.4)	20 (47.6)
[3]Positive only in Phase III	56 (13.5)	8 (14.3)	9 (16.1)	23 (41.1)	33 (58.9)
[4]Positive in Phase I & II	48 (11.6)	16 (33.3)	16 (33.3)	23 (47.9)	25 (52)
[5]Positive in Phase I & III	35 (8.4)	13 (37.1)	13 (37.1)	22 (62.9)	13 (37.1)
[6]Positive in Phase II & III	34 (8.2)	13 (38.2)	13 (38.2)	26 (76.4)	13 (38.2)
[7]Positive in the 3 Phases	20 (5.1)	10 (50)	10 (50)	17 (85)	3 (15)
[8] Total Carriers	241 (58.1)	77 (32)	78 (32.4)	133 (32)	108 (26)

[1]positive only in phase I, negative in phase II and III; [2]positive only in phase II, negative in phase I and III; [3]positive only in phase III, negative in phase I and II; [4]positive in phase I and II, negative in phase III; [5]positive in phase I and III, negative in phase II; [6]positive in phase II and III, negative in phase I; [7]positive in the 3 phases, cases carried *S. pneumoniae* in each phase; [8]at least carried *S. pneumoniae* once during the whole study period

Table 5. Vaccine and non-vaccine serotypes in each phase for centers of the Ajlun governorate.

Centers	Vaccine serotypes coverage at phase I				Vaccine serotypes coverage at phase II				Vaccine serotypes coverage at phase III			
	7v PCV (n; %)	10v PCV (n; %)	13v PCV (n; %)	Non-vaccine serotypes (n; %)	7v PCV (n; %)	10v PCV (n; %)	13v PCV (n; %)	Non-vaccine serotypes (n; %)	7v PCV (n; %)	10v PCV (n; %)	13v PCV (n; %)	Non-vaccine serotypes (n; %)
All centers	35;32.1	35;32.1	54;50.5	54;49.5	42;33.9	42;33.9	60 48.4	64; 51.6	18;14.8	19;15.6	45;36.9	77; 63.1
Ajlun center	2;33.3	2;33.3	4;66.7	2;33.3	3; 42.9	3; 42.9	4; 57.1	3; 42.9	0; 0	0; 0	2; 25	6; 75
Alhashmia	1;33.3	1;33.3	2;66.7	1;33.3	1; 33.3	1; 33.3	2; 66.7	1; 33.3	1;33.3	1; 33.3	2; 66.7	1; 33.3
Anjara	8;30.8	8;30.8	8;30.8	18;69.2	11;29.7	11;29.7	18;48.6	19; 51.4	4;17.4	4;17.4	7; 30.4	16; 69.6
Arjan	3;37.5	3;37.5	5;30.8	3;37.5	3;60	3; 60	5; 100	0; 0	2; 40	2; 40	3; 60	2; 40
Baaoon	1;25	1;25	2;50	2;50	2;66.7	2; 66.7	3; 100	0; 0	1; 25	1; 25	1; 25	3; 75
Ebben	3;30	3;30	5;50	5;50	2;20	2; 20	4; 40	6; 60	2;13.3	2; 13.3	6; 40	9; 60
Ein Janna	4;50	4;50	6;75	2;25	3;42.9	3; 42.9	5; 71.4	2; 28.6	1; 10	1; 10	3; 30	7; 70
Eshtafena	0;0	0;0	0;0	2;100	1;20	1; 20	2; 40	3; 60	1; 50	1; 50	1; 50	1; 50
Halawa	0;0	0;0	2;28.6	5;72.4	1;20	1; 20	3; 60	2; 20	0; 0	0; 0	2; 50	2; 50
Kafranjeh	10;37	10;37	17;63	10;37	9;34.6	9; 34.6	13; 50	13; 50	3;11.5	4; 15.4	9; 34.6	17; 65.4
Rajeb	1;100	1;100	1;100	0;0	0;0	0; 0	1; 50	1; 50	2; 50	2; 50	2; 50	2; 50
Sakhra	2;28.6	2;28.6	2;28.6	5;72.4	6; 42.9	6; 42.9	9; 64.3	5; 35.7	1; 5.6	1; 5.6	5;27.8	13; 72.2

Table 6. Macrolide resistant phenotypes and genotypes for all cumulative study isolates.

Macrolide Resistance phenotypes and genotypes	n (%)
M-phenotype	57 (48.3)
$cMLS_B$ phenotype	60 (50.8)
$iMLS_B$ phenotype	1 (0.9)
erm(B) genotype	59 (50.0)
mef(A) genotypes	53 (44.9)
erm(B) & *mef*(A) genotypes	2 (1.7)
Others	4 (3.4)

Table 7. Impact of vaccination with PCV7 on carriers with vaccine serotypes and the possible serotype shifts 3 months after last vaccination.

Center	Number of carriers having PCV7 serotypes	Number and rate of carriers no longer harboring PCV7 serotypes after last vaccination	Number of carriers with shifts from 19F to 19A and from 6B to 6A
Ajlun	4	4 (100%)	0
Alhashmia	3	2 (66%)	2
Anjara	21	17 (81%)	2
Arjan	5	3 (60%)	0
Baaoon	3	2 (66.7%)	0
Ebben	6	4 (66.7%)	1
Ein Janna	5	4 (80%)	1
Eshtafena	2	1 (50%)	0
Halawa	1	1 (100%)	0
Kafranjeh	17	14 (82.4%)	0
Rajeb	3	1 (33.3%)	0
Sakhra	5	4 (80%)	1
Total	75	57 (76%)	7

Table 8. Multidrug resistant (*MDR) isolates detected in carriers at the 3 phases of the study.

Phase	Number (%)
Phase I (n=109)	43 (39.5%)
Phase II (n= 124)	60 (48.4%)
Phase III (n= 122)	57 (46.7%)

*****MDR**=** isolates resistant to 3 or more classes of antimicrobial agents

4. Discussion

Nasopharynx is the usual source of pneumococci for studying the carriage rate (Malfroot *et al*., 2004). To our knowledge, this is the first study in Jordan to investigate the impact of vaccination with PCV7 on pneumococcal carriage and resistance among infants in Jordan. Two previous studies described pneumococcal carriage in Jordan; the first showed a carriage rate of 55.1% from Wadi Al Seer (Amman, Jordan), with a PCV7 and PCV13 coverage rates of 52.3% and 58.5%, respectively (Al-Lahham and Van der Linden, 2014). The second study showed a carriage rate of 33.8% from children below five years of age attending the pediatric clinic of a major hospital in northern Jordan (Swedan *et al*., 2016). This study demonstrated that the total carriage rate of *S. pneumoniae* among infants in the governorate of Ajlun was 58.1%. Lee *et al*., (2001) investigated carriage rate of pneumococci in 4963 Asian children aged below five years from eleven countries (Lee *et al*., 2001). The results of the study showed the following rates: Philippines (32.6%), China (37.5%), India (43.2%), Thailand (40.6%), Taiwan (15.3%), and Saudi Arabia (9.0%). Similar carriage rates were obtained from Brazil (55%) (Marchese and Schito, 2007), Guatemala (59.1%) (Marchisio *et al*., 2002), and in Kampala Uganda (62%) (Mera, 2005). The observed high rate of pneumococcal colonization in Ajlun can be attributed to history of the sicknesses, low age, viral infections, history of the consumption of antimicrobials before DCC attendance, and low income. The differences in carriage rates worldwide were related to certain socio-

economic conditions, including housing, access to health care, poor hygiene, family size, overcrowded living conditions, day-care contact, and the number of siblings (García-Rodríguez, 2002). Previous studies reported that attendance of day care is the main factor causing the increase in the *S. pneumoniae carriage rate* (Huang and Fang, 2004). The continuous surveillance of the antimicrobial susceptibility patterns of *S. pneumoniae* has become increasingly important, because of the rapid emergence of drug-resistant strains worldwide (Goyal *et al*., 2007). Consumption of antimicrobials prior to DCC visits could have contributed to the selection of resistant strains (Montanari *et al*., 2003). Antimicrobial susceptibility testing of the *S. pneumoniae* isolates revealed alarming rates of resistance to penicillin, erythromycin, and occurrence of multidrug-resistant (MDR) isolates. Rates of resistant *S. pneumoniae* isolates in Ajlun were higher than those for clinical isolates from Singapore, Sri Lanka, and Taiwan (Lee *et al*., 2001). The high rates of resistance to different classes of antimicrobial agents in *S. pneumoniae* in this study are presumably a consequence of the unregulated consumption and misuse of antimicrobials by the Jordanian population (Al-Bakri, 2005). Otoom *et al*., (2002) reported that antimicrobial agent prescriptions in Jordan at different health centers ranged between 46.7% to 83.3%; these rates are very high compared to rates in many other parts of the world (Otoom, 2002). Local information on capsular types of *S. pneumoniae* causing diseases in young children is highly important to guide the production of effective conjugate vaccines. Our results showed that 241 out of 245 infants were carriers over the whole year, The most prevalent serotypes among the carriers were 6A and 19F at 12.0% each. Similar serotypes have been reported in children of Kuwait, where 19F accounted for 9.8% of total serotypes (Ahmed *et al*., 2000). A study by Marchisio *et al*. (2002) in Italy, found an *S. pneumoniae* carriage rate of 8.6% with the following serotypes (3, 19F, 23F, 19A, 6B, and 14), and that most of pneumococcal isolates (69.4%) were resistant to one or more antimicrobial classes (Marchisio *et al*., 2002). Children at day-care centers in Belgium, aged 3-36 months, had a 21% *S. pneumoniae* carriage rate with the main serotypes being 19F (27.3%), 6B (20.2%), 23F (19.2%), 19A (10.1%), 6A (7.1%), and 14 (5.1%) (Malfroot *et al*., 2004). Prevenar, the 7-valent pneumococcal conjugate vaccine (PCV7) and the new 13-valent pneumococcal conjugate vaccine (PCV13) are used routinely in the National Immunization Program of at least seventy countries worldwide. The results of this study show that 241 out of 415 infants were carriers during at least one of the study phases, with a coverage of PCV7 serotypes of 27.8%, and a coverage of PCV13 serotypes of 49.8%. Around the world, the highest coverage for PCV7 has been reported in the USA, Canada, and Australia (80–90%), followed by Europe and Africa (70–75%), whereas in Latin America and Asia the coverage rates were 65% and 50%, respectively (Hausdorff *et al*., 2005). A retrospective review study in the USA was conducted by Walls *et al*. (2015) using the pediatric reports of 31,738 kids aged between 1-4 years and collected from the National Inpatient Database with complications of meningitis, mastoiditis, periorbital cellulitis, and Bezold abscesses due to *S. pneumoniae* diagnoses. A significant decrease in the incidence of several complications was noticed after the introduction of the PCV7 vaccine, and also when comparing these findings to the predicted incidence calculations if the vaccine was not administered. These findings showed a significant increase in the cost to provide care for each of the described conditions ($P < 0.05$), and resulted in a measurable reduction in the head and neck complications associated with *S. pneumoniae* (Walls *et al*., 2015).

A 15-year retrospective study was conducted for the years 2000 to 2014 by Soto-Noguerón, *et al*. (2015) on Mexican infants aged ≤60 days and having invasive and non-invasive pneumococcal infections. It showed that 40.5% of the Mexican infants had pneumonia followed by meningitis (29.4%), septicemia (16.7%), and other clinical presentations, including otitis media and conjunctivitis (13.5%). The study also showed that serotypes 15A/B had increased after the introduction of PCV7, and that serotype 19A was isolated most frequently with pneumonia and meningitis cases only after the introduction of PCV7, and that it displayed a high resistance to penicillin (Soto-Noguerón *et al*., 2015). In the current study, PCV was introduced in the private sector but not in the National Immunization Programme of Jordan. Furthermore, most of the families that vaccinated their children voluntarily were found to be of good income, and are more likely to be residing in Amman, the capital of Jordan. Vaccination with PCV7 in this study was effective in eradicating 76% of vaccine serotypes three months after the last vaccination. Finally, the researchers are aware that the carriage patterns may vary between communities and that it is possible that the serotype distribution and resistance patterns described here may not be representative of all the infant population of Jordan. An obstacle to the eradication of pneumococcal diseases in children is the inability to include more antigens in the conjugated formulations from the 92 serotypes of *S. pneumoniae*. Knowledge about the regional distribution of the pneumococcal capsular types, antimicrobial susceptibility, continuous prevalence studies, and incidence rates of both pneumococcal meningitis and bacteremia in children, is very essential for the future development of effective vaccine strategies and treatment protocols.

Acknowledgements

The authors thank Natasha from the National Reference Center for Streptococci in Germany for the excellent technical assistance. The study was supported in part by a grant from Wyeth (now Pfizer). We acknowledge also the financial support of the Deanship of Scientific Research at the German Jordanian University in 2009. The assistance of the National Reference Center for Streptococci in Aachen, Germany in the serotyping and resistance analysis is highly appreciated. We also thank the Ministry of Health for providing the team for the collection of samples at the DCCs.

References

Adegbola RA, DeAntonio R, Hill PC, Roca A, Usuf E, Hoet B, and Greenwood BM. 2014. Carriage of *Streptococcus pneumoniae* and other respiratory bacterial pathogens in low and lower-middle income countries: a systematic review and meta-analysis. *PLoS One*, **9**: e103293.

Ahmed K, Martinez G, Wilson S, Yoshida R, Dhar R, Mokaddas E, Kohno S, Rotimi VO and Nagatake T. 2000. The prevalence and clonal diversity of penicillin-resistant *Streptococcus pneumoniae* in Kuwait. *Epidemiol Infect*, **125**: 573-81.

Al-Bakri AG, Bustanji Y and Yousef AM. 2005. Community consumption of antibacterial drugs within the Jordanian population: sources, patterns and appropriateness. *Int J Antimicrob Agents.* **26**: 389-95.

Al-Lahham A and Van der Linden M. 2014. *Streptococcus pneumoniae* carriage, resistance and serotypes among Jordanian children from Wadi Al Seer District, JORDAN. *Inter Arab J Antimicrob Agents*. **4**: 1-8.

Amodio E, Costantino C, Boccalini S, Tramuto F, Maida CM and Vitale F. 2014. Estimating the burden of hospitalization for pneumococcal pneumonia in a general population aged 50 years or older and implications for vaccination strategies. *Hum Vaccin Immunother*. **10**: 1337-42.

Bogaert D, van Belkum A, Sluijter M, Luijendijk A, de Groot R, Rumke HC, Verbrugh HA and Hermans PW. 2004. Colonisation by *Streptococcus pneumoniae* and *Staphylococcus aureus* in healthy children. *Lancet*, **363**: 1871-2.

CLSI. **Performance Standards for Antimicrobial Susceptibility Testing. 27**th ed. CLSI supplement M100. Wayne, PA: Clinical and Laboratory Standard Institute ; 2017.

Farrell DJ, Morrissey I, Bakker S and Felmingham D. 2001. Detection of macrolide resistance mechanisms in *Streptococcus pneumoniae* and *Streptococcus pyogenes* using a multiplex rapid cycle PCR with microwell-format probe hybridization. *J Antimicrob Chemother*. **48**: 541-4.

File TM. 2006. Clinical implications and treatment of multiresistant *Streptococcus pneumoniae* pneumonia. *Clin Microbiol Infect.* **12** Suppl **3**: 31-41.

García-Rodríguez JA and Fresnadillo Martínez MJ. 2002. Dynamics of nasopharyngeal colonization by potential respiratory pathogens. *J Antimicrob Chemother.* **S2**: 59-73.

Goyal R, Singh NP, Kaur M and Talwar V. 2007. Antimicrobial resistance in invasive and colonising *Streptococcus pneumoniae* in North India. *Indian J Med Microbiol*. **5**: 256-259.

Hausdorff WP, Feikin DR and Klugman KP. 2005. Epidemiological differences among pneumococcal serotypes. *Lancet Infect Dis*, **5**: 83-93.

Huang WH and Fang SY. 2004. High prevalence of antibiotic resistance in isolates from the middle meatus of children and adults with acute rhinosinusitis. *Am J Rhinol*. **18**: 387-91.

Jacobs MR and Dagan R. 2004. Antimicrobial resistance among pediatric respiratory tract infections: clinical challenges. *Semin Pediatr Infect Dis*. **15**: 5-20.

Krone CL, Wyllie AL, van Beek J, Rots NY, Oja AE, Chu ML, Bruin JP, Bogaert D, Sanders EA and Trzcinski K. 2015. Carriage of *Streptococcus pneumoniae* in aged adults with influenza-like-illness. *PLoS One*, **10**: e0119875.

Lee NY, Song JH, Kim S, Peck KR, Ahn KM, Lee SI, Yang Y, Li J, Chongthaleong A, Tiengrim S, Aswapokee N, Lin TY, Wu JL, Chiu CH, Lalitha MK, Thomas K, Cherian T, Perera J, Yee TT, Jamal F, Warsa UC, Van PH, Carlos CC, Shibl AM, Jacobs MR and Appelbaum PC. 2001. Carriage of antibiotic-resistant pneumococci among Asian children: a multinational surveillance by the Asian Network for Surveillance of Resistant Pathogens (ANSORP). *Clin Infect Dis*, **32**: 1463-9.

Ludwig E. 2013. [Pneumococcal infections -- clinical features, prevention and current therapy. Interview with Dr. Endre Ludwig by Anna Radnai]. *Orv Hetil*. **154**: 118-20.

Ludwig E, Bonanni P, Rohde G, Sayiner A and Torres A. 2012. The remaining challenges of pneumococcal disease in adults. *Eur Respir Rev*. **21**: 57-65.

Malfroot A, Verhaegen J, Dubru JM, Van Kerschaver E and Leyman S. 2004. A cross-sectional survey of the prevalence of *Streptococcus pneumoniae* nasopharyngeal carriage in Belgian infants attending day care centres. *Clin Microbiol Infect*. **10**: 797-803.

Marchese A and Schito GC. 2007. Recent findings from multinational resistance surveys: are we 'PROTEKTed' from resistance?. *Int J Antimicrob Agents*. **29** Suppl 1: S2-5.

Marchisio P, Esposito S, Schito GC, Marchese A, Cavagna R and Principi N. 2002. Nasopharyngeal carriage of *Streptococcus pneumoniae* in healthy children: implications for the use of heptavalent pneumococcal conjugate vaccine. *Emerg Infect Dis*. **8**: 479-84.

Mera R. 2005. Predicting the future *Streptococcus pneumoniae* resistance landscape. *Curr Opin Pharmacol*. **5**: 459-64.

Montanari MP, Cochetti I, Mingoia M and Varaldo PE. 2003. Phenotypic and molecular characterization of tetracycline- and erythromycin-resistant strains of *Streptococcus pneumoniae. Antimicrob Agents Chemother*. **47**: 2236-41.

Montanari MP, Mingoia M, Giovanetti E and Varaldo PE. 2001. Differentiation of resistance phenotypes among erythromycin-resistant Pneumococci. *J Clin Microbiol*, **39**: 1311-5.

Otoom S, Batieha A, Hadidi H, Hasan M and Al-Saudi K. 2002. Evaluation of drug use in Jordan using WHO prescribing indicators. *East Mediterr Health J.* **8**: 537-43.

Pallares R, Fenoll A and Linares J. 2003. The epidemiology of antibiotic resistance in *Streptococcus pneumoniae* and the clinical relevance of resistance to cephalosporins, macrolides and quinolones. *Int J Antimicrob Agents*, **22** Suppl 1: S15-24; discussion S25-6.

Reinert RR, Franken C, van der Linden M, Lutticken R, Cil M and Al-Lahham A. 2004. Molecular characterisation of macrolide resistance mechanisms of *Streptococcus pneumoniae* and *Streptococcus pyogenes* isolated in Germany, 2002-2003. *Int J Antimicrob Agents*. **24**: 43-7.

Reinert RR, Jacobs MR, Appelbaum PC, Bajaksouzian S, Cordeiro S, van der Linden M and Al-Lahham A. 2005. Relationship between the original multiply resistant South African isolates of *Streptococcus pneumoniae* from 1977 to 1978 and contemporary international resistant clones. *J Clin Microbiol*. **43**: 6035-41.

Reinert RR, Lutticken R, Bryskier A and Al-Lahham A. 2003. Macrolide-resistant *Streptococcus pneumoniae* and *Streptococcus pyogenes* in the pediatric population in Germany during 2000-2001. *Antimicrob Agents Chemother*. **47**: 489-93.

Saha SK, Baqui AH, Darmstadt GL, Ruhulamin M, Hanif M, El Arifeen S, Santosham M, Oishi K, Nagatake T and Black RE. 2003. Comparison of antibiotic resistance and serotype composition of carriage and invasive pneumococci among Bangladeshi children: implications for treatment policy and vaccine formulation. *J Clin Microbiol*. **41**: 5582-7.

Said MA, Johnson HL, Nonyane BA, Deloria-Knoll M, O'Brien KL, Agedd Adult Pneumococcal Burden Study Team, Andreo F, Beovic B, Blanco S, Boersma WG, Boulware DR, Butler JC, Carratala J, Chang FY, Charles PG, Diaz AA, Dominguez J, Ehara N, Endeman H, Falco V, Falguera M, Fukushima K, Garcia-Vidal C, Genne D, Guchev, F. Gutierrez, S. S. Hernes, A. I. Hoepelman, U. Hohenthal, N. Johansson, V. Kolek, R. S. Kozlov, T. L. Lauderdale, I. Marekovic, M. Masia, M. A. Matta, O. Miro IA, Murdoch DR, Nuermberger E, Paolini R, Perello R, Snijders D, Plecko V, Sorde R, Stralin K, van der Eerden MM, Vila-Corcoles A and Watt JP. 2013. Estimating the burden of

pneumococcal pneumonia among adults: a systematic review and meta-analysis of diagnostic techniques. *PLoS One.* **8**: e60273.

Soto-Noguerón A, Carnalla-Barajas MN, Solórzano-Santos F, Arrendondo-García JL, Arzate-Barbosa P, Tinoco-Favila JC, Anzurez-Gutiérrez A and Echániz-Aviles G. 2015. *Streptococcus pneumoniae* as cause of infection in infants less than 60 days of age: serotypes and antimicrobial susceptibility. *Int J Infect Dis*. **42**: 69-73.

Swedan SF, Hayajneh WA and Bshara GN.2016. Genotyping and serotyping of macrolide and multidrug resistant *Streptococcus pneumoniae* isolated from carrier children. *Indian J Med Microbiol.* , **34**: 159-165.

Volonakis K, Souli M, Kapaskelis A, Baziaka F, Grammelis V, Ziakas PD and Giamarellou H. 2006. Evolution of resistance patterns and identification of risk factors for *Streptococcus pneumoniae* colonisation in daycare centre attendees in Athens, Greece. *Int J Antimicrob Agents*. **28**: 297-301.

Walls A, Pierce M, Krishnan N, Steehler M and Harley EH Jr. 2015. Pediatric Head and Neck Complications of *Streptococcus pneumoniae* before and after PCV7 Vaccination. *Otolaryngol Head Neck Surg*. **152**: 336-41.

Wardlow TM, Johansson EW, Hodge MJ, World Health Organization, UNICEF. 2006. Pneumonia: The forgotten Killer of Children.

Williams JD. 2002. *Streptococcus pneumoniae* still going strong. *Int J Antimicrob Agents.* **20**: 75.

JJBS

Volume 11, Number 2,June 2018
ISSN 1995-6673
Pages 163 - 169

Jordan Journal of Biological Sciences

The Protective Effect of Polyphenol-Rich Extract of *Syzygium cumini* Leaves on Cholinesterase and Brain Antioxidant Status in Alloxan-Induced Diabetic Rats

Basiru O. Ajiboye[1,*], Oluwafemi A. Ojo[1], Olivia S. Akuboh[1], Marry A. Okesola[1], Olajumoke T. Idowu[1], Babatunji E. Oyinloye[1] and Justina Y. Talabi[2]

[1]*Department of Chemical Sciences;* [2]*Department of Human Nutrition and Dietetics, Afe Babalola University, Ado-Ekiti, Ekiti State, Nigeria*

Received September 21, 2017; Revised October 23, 2017; Accepted November 4, 2017

Abstract

Syzygium cumini leaves are used locally especially in Nigeria for the treatment\ management of diabetes mellitus and Alzheimer's disease. This study was designed to investigate the effects of polyphenols extracted from *Syzygium cumini* leaves on the occurrence of oxidative stress in the brain of rats with diabetes, which can trigger Alzheimer's disease by determining both *in vitro* and *in vivo* cholinesterase, the antioxidant defense system, and the extent of oxidative damage. The effect of polyphenols extracted from *Syzygium cumini* leaves was investigated on *in vitro* cholinesterase. Thereafter, the extract (400 mg/kg body weight) of both free and bound polyphenols was administered orally to alloxan-induced rats, and the effect were monitored on *in vivo* cholinesterase, superoxide dismutase, catalase, glutathione peroxidase, reduced glutathione, lipid peroxidation and hydroperoxides. The extract demonstrated inhibitory effects against *in vitro* cholinesterase. A significant reduction in the cholinesterase activities increased the activities of superoxide dismutase, catalase, glutathione peroxidase, and reduced glutathione. A reduction in lipid peroxidation and hydroperoxide concentrations was observed in the brain of diabetic rats treated with polyphenols extracted from *Syzygium cumini* leaves. This study suggests that the polyphenols of *Syzygium cumini* leaves have anti-Alzheimer and antioxidant boosters, as well as antiperoxidative activities. Therefore, the plant is recommended for both diabetic and Alzheimer's disease patients worldwide.

Keywords: *Syzygium cumini*, Diabetic mellitus, Antioxidant enzymes, Alzheimer's disease

1. Introduction

Functional foods are characterized by bioactive constituents, including the polyphenolic compounds, which form a defensive mechanism against oxidative insults (Khan, 2012). These polyphenolic compounds are antioxidant, anti-diabetic, anti-cancer, anti-microbial, and anti-inflammatory. Also, polyphenols are an integral part of the human diet, and are available in functional foods that are utilized in alternative medicine (Kazeem *et al*., 2013). One among the key complications of diabetes mellitus is the neurological disturbance in the central nervous system. Pari and Latha (2004) reported that cognitive deficits and the morphological and neuro-chemical alterations demonstrate neurological complications in patients with diabetes mellitus.

Persistent hyperglycaemia as well as the high levels of reactive oxygen species (ROS) could trigger central nervous system disorders, particularly Alzheimer's disease. Alzheimer's disease patients are characterized by an elevated activity of acetylcholinesterase (AChE) (EC 3.1.1.7) primarily in the brain, which is responsible for the breakdown of acetylcholine (ACh). According to Chang *et al*. (2014), ACh is a neurotransmitter that plays a significant role in the correct functioning of the central cholinergic system. Also, butrylcholinesterase (BChE) (EC 3.1.1.8) is responsible for the breakdown of certain drugs, toxins (before reaching the nerves), including the choline esters used in anaesthesia. BChE is found in a higher level in the Alzheimer's disease plaques than in the plaques of age-related non-demented brains (Schneider, 2001). Currently, more than 30 million individuals suffer from this disease, and it has been projected that by 2050 more than 115 million people will have dementia, if necessary actions were not taken (Querfurth and LaFerla, 2010).

Moreover, the neuronal network, particularly of the brain, is more liable to oxidative damage because of the elevated levels of polyunsaturated lipids in neuronal cell membranes. The high oxygen consumption, the high metabolic rate of transitional metals, and the poor antioxidative defense contribute to the prevalence of Alzheimer's disease (Lane *et al*., 2006). Therefore, antioxidant defenses of the brain are unpretentious. Uddin *et al*. (2016) define antioxidants as exogenous or endogenous substances that prevent oxidation, and act against the oxidative stress associated with deleterious effects on the cellular system. They are also coupled with

* Corresponding author. e-mail: ajiboyebo@abuad.edu.ng, bash1428@yahoo.co.uk.

free radical scavenging preventing free radical chain-reactions and improving the antioxidant status in Alzheimer's disease patients (Uttara *et al*., 2009). The antioxidant defense enzymes in the brain, including catalase (EC 1.11.1.6), superoxide dismutase (EC 1.15.1.1), and glutathione peroxidase (GSH-Px) (EC 1.11.1.9) among others, play an important role to prevent the progression of free radical mediated oxidative stress, (Sharma *et al*., 2013).

Recently, the use of functional foods is increasing worldwide probably because of their no or less side effects in comparison with modern available drugs. Several plants have been documented in this regard. One example is *Syzygium cumini* (L.) Skeels (popularly called jamun in India, and black plum in Europe etc.) [Fam.: Myrtaceae; Syn.: *Syzygium jambolanum* (Lam.) DC, *Eugenia jambolana* Lam., *Eugenia cumini* (L.) Druce] which has been used for years by the Indian Ayurvedic medicine practitioners for various therapeutic reasons (Sanches *et al.,* 2016). Therefore, this study was conducted to evaluate the role of the polyphenolic-rich extract of *Syzygium cumini* leaves on cholinesterase and the antioxidant efficacies on the brains of rats with alloxan-induced diabetes.

2. Materials and Methods

2.1. Drugs and Chemicals

All the drugs and chemicals used in this study were obtained from Sigma Chemical Company Inc., St. Louis, Mo, USA. Also, all reagents used were of analytical grades.

2.2. Plant Material

Fresh leaves of *Syzygium cumini* were collected from the vicinity of the Zamani College in Kaduna, Nigeria in September, 2016. The plant was identified and authenticated at the Department of Botany of Ahmadu Bello University in Zaria. The leaves were washed under tap water, and were rinsed with distilled water. The leaves were then air-dried for seven days using paper towels. Thereafter, they were ground into powder by a blender, and were stored at 10 ^{0}C to be used later in the analysis.

2.3. The Extraction of Phenolics

2.3.1. The Extraction of Free Phenolics

One hundred grams of powdered *Syzygium cumini* leaves were extracted with 80 % acetone (1:5 w/v), and were filtered using Whatman (Number 4) filter paper. The filtrate was then evaporated by a rotary evaporator under vacuum at 45 ^{0}C until almost 90 % of the filtrate had been evaporated and lyophilized to obtain a dry extract. The extract was then kept at -4 ^{0}C for subsequent analyses while the residue was kept for the extraction of bound phenolics (Chu *et al.,* 2002).

2.3.2. The Extraction of Bound Phenolics

The residue obtained from the extraction of free phenolics was flushed with nitrogen and hydrolyzed with 20 mL of 4 M NaOH solution at room temperature (24 ±1 ^{0}C) for a one-hour shaking. After that, the pH of the mixture was adjusted to two using concentrated HCl. Then the bound phytochemicals were extracted with ethylacetate six times. The ethylacetate extracts were evaporated to dryness at 45 ^{0} C using a rotary evaporation as described by Chu *et al.* (2002).

2.4. Animals

Male Wistar albino rats (seven weeks old), weighing between 150 to 170 gram were obtained from the Central Animal House of Afe Babalola University in Ado-Ekiti, Ekiti State in Nigeria. The animal experiments were approved by the ABUAD Animal Ethical Committee. The animals were acclimatized for fourteen days. They were fed with normal rat pellet chow and were kept under constant light and dark cycles for twelve hours, with an environmental temperature of 21 to 23 ^{0}C.

2.5. Induction of Diabetes

Diabetes was induced in the albino rats by a single intraperitoneal injection of freshly-prepared alloxan of 150 mg/kg body weight in normal saline. Forty-eight hours (two days) following the alloxan induction, blood samples were obtained from the tips of the rats' tails and the fasting blood glucose levels were determined using the OneTouch Ultra glucometer (LifeScan, USA) to confirm diabetes. The diabetic rats exhibiting blood glucose levels greater than or equal to 200 mg/mL were used in this study (Akanksha *et al*., 2010; Ajiboye and Ojo, 2014).

2.6. Animal Grouping

Only male rats were used in this study to avoid differences in the hormonal actions and to have a proper comparison within the same-sex group. Also, using both sexes in one group may lead to the impregnation of the female rats by male rats, which might affect the experimental outcome. Accordingly, a total of thirty male Wistar albino rats were randomly divided into five groups with six rats in each and were treated as follows:

Group 1: normal rats administered with distilled water;
Group 2: alloxan-induced diabetic rats (without treatment);
Groups 3 and 4: alloxan-induced diabetic rats administered with 400 mg/kg of the free and bound polyphenolic extract of *Syzygium cumini* leaves respectively.
Group 5: alloxan-induced diabetic rats administered with 5 mg/kg of metformin.

The extract was suspended in distilled water, and was orally administered to rats daily for fourteen days by an orogastric tube. In addition, 400 mg/kg of both free and bound phenolics was used based on the oral glucose tolerance test (OGTT) previously determined by the authors.

2.7. The Preparation of Tissue Homogenates

The experiment lasted for fourteen days, after which the rats were sacrificed by cervical dislocation. Their brains were immediately removed, and rinsed in ice-cold 1.15% KCl, then blotted and weighed. The brains were then minced with scissors in three volumes of ice-cold 100 mM potassium phosphate buffer, pH 7.4, and were homogenized in a teflon glass homogenizer. The homogenates were centrifuged for ten minutes at 12,000 × *g* to yield a pellet that was discarded. A low speed supernatant (S1) was used to assess the activities of different enzyme studied assays (Belle *et al.* 2004).

2.8. In vitro Determination of Acetylcholinesterase and Butyrylcholinesterase Inhibitory Activities

These were determined by a modified colorimetric method of Ellman *et al*. (1961). The AChE activity was determined in a reaction mixture containing 200 µL of a solution of AChE (0.415 U/mL in 0.1 M phosphate buffer, pH 8.0), 100 µL of a solution of 5, 5'-dithiobis (2-nitrobenzoic acid) (3.3 mM DTNB in 0.1 M phosphate buffered solution of pH 7.0, containing 6 mM $NaHCO_3$), the plant extract and 500 µL of phosphate buffer (pH 8.0). After incubation for twenty minutes at 25 ^{0}C, 100 µL of 0.05 mM acetylthiocholine iodide solution was added as the substrate, and AChE activity was determined as changes in the absorbance reading at 412 nm for three minutes at 25 ^{0}C using a spectrophotometer. Also, 100 µL of butyrylthiocholine iodide was used as a substrate to assay butyrylcholinesterase activity, while all the other reagents and conditions being the same. The AChE and BChE inhibitory activities were expressed as percentage inhibition.

2.9. Determination of in vivo Acetylcholinesterase and Butyrylcholinesterase Inhibitory Activities

The method of Ingkaninan *et al.* (2003) was employed in this determination. Briefly, 140 µL of sodium phosphate (0.1 M, pH 6.8) was pipette into a test tube, and 20 µL of the brain homogenate and 20 µL of acetylcholinesterase or butyrylcholinesterase (0.09 unit/mL) were added as the case may be. The mixture was incubated for fifteen minutes at room temperature. Thereafter, 10 µL of 10 mM DTNB and 10 µL of acetylthiocholine chloride (14 mM) or s-butyrylthiocholine chloride (substrate) as the case may be were added. Finally, the mixture was allowed to stand for fifteen minutes, and the absorbance was read at 417 nm using a microplate reader.

2.10. Determination of Catalase (CAT) Activity

The catalase activity of the brain homogenates was determined according to the method described by Sinha (1972). One millimeter of the supernatant fraction of the brain homogenate was mixed with 19 mL distilled water to give a 1:20 dilution. The assay mixture contained 4 mL of hydrogen peroxide solution (800 mmol) and 5 mL of phosphate buffer at pH 7.0 in a 10-mL flat bottom flask. Thereafter, 1 ml of the properly diluted sample was rapidly mixed with the reaction mixture by a gentle swirling motion at room temperature. Then 1 mL portion of the reaction mixture was withdrawn and blown into 2 mL dichromate/acetic acid reagent at 60-second intervals. The hydrogen peroxide contents of the withdrawn sample were determined by reading the absorbance at 570 nm.

2.11. Determination of the Superoxide Dismutase (SOD) Activity

The SOD activity was determined according to the method described by Misra and Fridovich (1972). An aliquot of the brain homogenate was added to 2.5 mL of 0.05 M carbonate buffer (pH 10.2) to equilibrate in the spectrophotometer. The reactions were initiated by the addition of 0.3 mL freshly prepared 0.3 mM adrenaline to the mixture which was quickly mixed by inversion. The reference cuvette contained 2.5 mL buffer, 0.3 mL of adrenaline, and 0.2 mL of water. The increase in absorbance at 480 nm was monitored every thirty seconds.

2.12. Determination of Reduced Glutathione Concentration

The reduced glutathione (GSH) level was determined using Ellman's reagent, 5,5'-dithio-bis-2-nitrobenzoic acid (DTNB) as described by Jollow *et al*. (1974). This method is based on the development of a relatively-stable yellow complex formed as a result of the reaction between Ellman's reagent and free sulphydryl groups. The chromophoric product, 2–nitro–5– thiobenzoic acid, resulting from the reaction of Ellman's reagent with GSH. The absorbance of the complex formed was read at 412 nm, which is proportional to the level of GSH in the brain homogenate sample. Thereafter, the activity of GSH was determined.

2.13. Glutathione Peroxidase (GPx) Assay

This assay was carried out using the Rotruck *et al.* (1973) method. The reaction mixture containing 500 mL phosphate buffer, 100 mL sodium azide, 200 mL of GSH, and 100 mL of H_2O_2 was added to 500 mL of the brain homogenate, after which 600 mL of the sample (brain homogenate) was added and thoroughly mixed. The whole reaction mixture was incubated at 37 ^{0}C for three minutes, after which 0.5 mL TCA (trichloroacetic acid) was added and thereafter centrifuged at 3000 g for 5 minutes. Subsequently, to 1 mL of each of the supernatants, 2 mL of K_2HPO_4 and 1 mL DTNB (Ellman's Reagent) was added and the absorbance was read at 412 nm against a blank.

2.14. Determination of Lipid Peroxidation

Lipid peroxidation was determined according to the method described by Adam-Vizi and Seregi (1982). An aliquot of 0.4 mL of the brain homogenate was mixed with 1.6 mL of Tris-KCl buffer to which 0.5 mL of 30% TCA was added. Then 0.5 mL of 0.75% TBA was added and placed in a water bath for fort-five minutes at 80 ^{0}C. This was then cooled in ice, and centrifuged at 3000 g for five minutes. The clear supernatant was collected, and the absorbance was measured against a reference blank of distilled water at 532 nm. The malondialdehyde level was then calculated.

2.15. Determination of Hydroperoxides

The method described by Jiang *et al.* (1992) was used for this determination. Briefly, 0.1 mL of brain homogenate was added to 0.9 mL of Fox reagent (contained 88 mg of butylated hydroxytoluene, 7.6 mg of xylenol orange and 9.8 mg of ammonium ion sulphate were added to 90 mL of methanol and 10 mL of 250 mM sulphuric acid). This was then incubated at 37 ^{0}C for thirty minutes. The color developed was read at 560 nm calorimetrically. The hydroperoxides were expressed as mM/100 g tissue.

2.16. Data Analysis

Data were expressed as the mean of six replicates ± standard error of mean. The statistical evaluation of data was performed by SPSS (version 20). Using the one-way analysis of variance, followed by Dunett's post hoc test for multiple comparisons, values were considered statistically-significant at $p < 0.05$ (Zar, 1984).

3. Results

Figure 1 and 2 show the effects of the free and bound polyphenolic extract of *Syzygium cumini* leaves on the acetlycholinesterase and butyrylcholinesterase inhibitory activities respectively. The extracts demonstrated a significant increase ($p < 0.05$) in dose dependent manner in the inhibitory activities of acetlycholinesterase and butyrylcholinesterase. However, the bound polyphenolic extract of the *Syzygium cumini* leaves showed more inhibitory activities in dose dependent manner than the free polyphenolic extract on both cholinesterases.

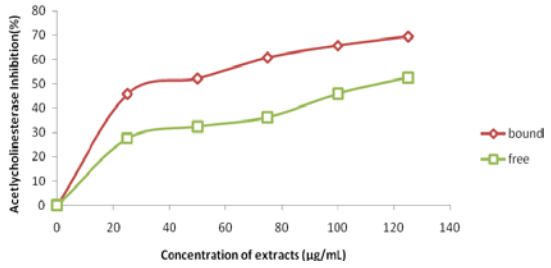


Figure 1. Effect of polyphenolic extract of Syzygium *cumini* leaves on acetylcholinesterase (AChE) activity *in vitro*. Each value is a mean three replicates ± standard error of mean.

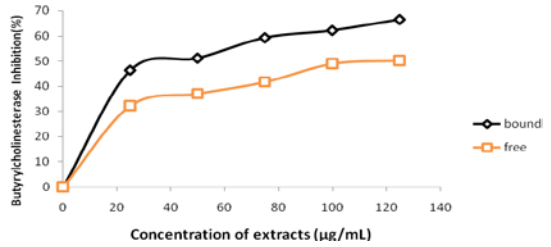


Figure 2. Effect of polyphenolic extract of Syzygium cumini leaves on butyrylcholinesterase (BChE) activity in vitro. Each value is a mean three replicates ± standard error of mean.

The brain of diabetic control rats demonstrated a significant ($p < 0.05$) increase in cholinesterases inhibitory activities when compared with normal control (Figure 3). But by the end of the fourteen-day treatment with the polyphenolic extract of the *Syzygium cumini* leaves, the activities of these enzymes were significantly reduced ($p < 0.05$), and compared favorably with normal control rats in the metformin-treated groups.

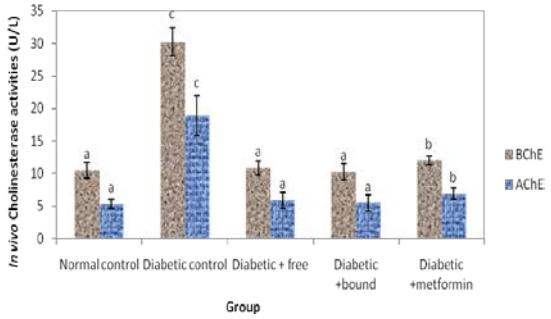


Figure 3. Effect of administration of polyphenols from *Syzygium cumini* leaves on *in vivo* cholinesterases activities in brain of normal and alloxan-induced diabetic rats. Each value is a mean of six replicates ± standard error of mean.

The activities of catalase (CAT), superoxide dismutase (SOD), reduced glutathione (GSH) and glutathione peroxidase (GPx) were significantly decreased ($p < 0.05$) in the diabetic control rats when compared with the normal rats (Table 1). Also, the treatment of diabetic rats with the free and bound polyphenolic extract of the *Syzygium cumini* leaves significantly increased ($p<0.05$) the activities of all the antioxidant enzymes studied and compared favorably with the normal control.

Table 1. Effect of administration of polyphenols from *Syzygium cumini* leaves on antioxidant enzymes activities (U/mg protein) in the brain of normal and alloxan-induced diabetic rats.

Group	Catalase (CAT)	Superoxide dismutase (SOD)	Glutathione Peroxidase (GPx)	Reduced glutathione (GSH)
Normal Control	86.23±3.56^a	43.56 ±4.80^a	64.78 ± 8.34^a	30.21 ±5.56^a
Diabetic control	15.23 ± 2.12^c	9.69 ± 2.12^c	12.89 ± 3.65^c	11.67 ±6.80^c
Diabetic + free polyphenols	84.89 ± 7.34^a	40.58 ±5.48^a	63.84 ± 5.29^a	28.76 ±9.24^a
Diabetic + bound polyphenols	85.65 ± 4.38^a	42.39 ± 6.28^a	63.97 ± 6.87^a	29.56 ±7.00^a
Diabetic + metformin	75.29 ± 2.14^b	35.38 ±3.45^b	57.86 ± 4.80^b	25.56 ±3.11^b

Values are mean ± SEM of six rats. Values down the vertical column carrying different superscripts for each parameters are significantly different at $p<0.05$.

There was a significant increase ($p < 0.05$) in the brain lipid peroxidation and hydroperoxides of the diabetic control rats in comparison to the normal control and metformin- treated diabetic rats (Table 2). The fourteen-day administration of the polyphenolic extract of the *Syzygium cumini* leaves on diabetic rats significantly decreased ($p < 0.05$) the level of lipid peroxidation and hydroperoxides in the brains of rats, compared favorably with normal control rats.

Table 2. Effect of administration of polyphenols from *Syzygium cumini* leaves on lipid peroxidation (MDA) and hydroperoxides concentrations in the brain of normal and alloxan-induced diabetic rats**.**

Group	MDA (X 10^9 mmol/mL)	Hydroperoxides (mM/100g tissue)
Normal control	1.34 ± 0.14^a	100.21 ± 4.28^a
Diabetic control	6.42 ± 0.34^c	150.45 ± 6.90^c
Diabetic + free polyphenols	1.36 ± 0.34^a	100.34 ± 5.23^a
Diabetic + bound polyphenols	1.35 ± 0.45^a	102.58 ± 7.32^a
Diabetic + metformin	2.13 ± 0.67^b	110.12 ± 4.21^b

Values are mean ± SEM of six rats. Values down the vertical column carrying different superscripts for each parameters are significantly different at $p<0.05$

4. Discussion

Persistent/chronic hyperglycaemia might cause an imbalance in the oxidative status of the nervous tissue. Thus, the resulting free radicals could injure the brain (a key organ in the nervous system) by a process referred to as peroxidative mechanism (Pari and Latha, 2004). This

might lead to a disease condition known as Alzheimer's diseases which suggested that continuous hyperglycemia in diabetes mellitus patients may interact with the acetylcholine/butyrylcholine receptors affecting the binding efficiency and leading to an increase in the AChE/BChE activity, and the decomposition of the higher levels of the neurotransmitter. According to Wacker *et al*. (2005) acetylcholine, the principal neurotransmitter of the cholinergic neurons, is one amongst many neurotransmitters responsible for neurodegenerative diseases. It is also related to cognitive functions concerned with learning and memory processes (Blockland, 1995). Moreover, cholinergic neurons correspond to about 25% of the brain cells, which are represented mainly by cortical and hippocampal neurons (Wacker, 2005). The synaptic cholinergic transmission depends on the acetylcholinesterase (AChE) activity, since this enzyme promotes the hydrolysis of the neurotransmitter acetylcholine in choline and acetic acid, resulting in the terminus of the transmission of the nervous impulse within the synapses (Taylor, 1996). During this study, the polyphenols extracted from the *Syzygium cumini* leaves demonstrated inhibitory activities of AChE and BChE in dose dependent manner (Figures 1 and 2). This is an indication that the extract can be useful in the ameliorating AChE/BChE activity, and may therefore serve as a neurotransmitter booster. Also, this was supported by the *in vivo* inhibitory activities of AChE and BChE (Figure 3), which was in agreement with the findings in Gill *et al.* (1991) and Gallegos *et al*. (2001).

In addition, the likely mechanism, by which the persistent hyperglycemia causes neurotoxicity, is probably by targeting the learning and memory processes of the brain by inhibiting the N-methyl-D-aspartate receptor (NMDAR), essential for hippocampus-mediated learning and memory (Akinyemi *et al*., 2015). Furthermore, Orhan *et al*. (2004) reported that the enhanced activities of AChE and BChE are detrimental to patients suffering from Alzheimer's disease. Accordingly, the consumption of polyphenols extracted from *Syzygium cumini* leaves can be very functional to health as it could restructure the neurodegeneration by inhibiting the AChE and BChE activities.

Moreover, the treatment with antioxidants is a promising approach for reducing the progression of Alzheimer's disease, associated with chronic hyperglycemia coupled with oxidative stress. Grundman *et al.* (2002) reported a link between antioxidants and reduced incidence of dementia. The vulnerability of the brain to oxidative stress induced by oxygen free radicals might be attributed to the utilization of one fifth of the total oxygen demand of the body; the brain is not enriched with enough antioxidant enzymes when compared with other organs (Bayes and Thrope, 1999). Antioxidant enzymes play an important role in the maintenance of physiological concentrations of oxygen and hydrogen peroxides by enhancing the dismutation of oxygen radicals and mopping up organic peroxides generated by the exposure to alloxan (Kazeem *et al*., 2013). Superoxide dismutase (SOD) may mop up superoxide radicals by converting them into H_2O_2 and oxygen while both CAT and GPx are useful in the elimination of H_2O_2 (Vincent *et al*., 2004). In this study, a significant reduction was noted in the activities of CAT, SOD and GPx (Table 1) in the brain of diabetic rats. This can be attributed to the rise in the generation of reactive oxygen species like superoxide and the hydroxyl radical by alloxan (Kaleem *et al*., 2006). Also, the free radicals produced may inactivate the activities of these antioxidant enzymes (Soon and Tan, 2002).Accordingly, this may be accountable for the dearth of antioxidant defenses in justifying the ROS mediated damage (Pari and Latha, 2005). The administration of polyphenols extracted from *Syzygium cumini* leaves enhanced the activities of these antioxidant enzymes probably by ameliorating the imbalance between the ROS production and the antioxidant enzyme-activity in diabetic rats. This may be responsible for the AChE and BChE inhibitory activities observed in Figure 3.

In the same context, GSH is an antioxidant and its reduction has been documented in diabetes mellitus, which may be attributed to its increased utilization by oxidative stress (Pari and Latha, 2004). In the current study, the decreased GSH activity was observed in diabetic rats, but at the end of the fourteen-day experimental period of the administration of the polyphenols extracted from the *Syzygium cumini* leaves, the brain GSH activity increased. The elevated levels can protect cellular proteins against oxidation through the glutathione redox cycle (Yu, 1994).

In addition, the elevated levels of MDA and hydroperoxides were observed in brains of diabetic rats, because the brain contains a high concentration of easily peroxidizable fatty acids (Carney *et al.,* 1991); this was in agreement with the present study (Table 2). However, the administration of polyphenols extracted from *Syzygium cumini* leaves reversed their concentrations. This may be attributed to the free radical scavenging ability of the extract. The extract was found to be anti-cholinesterase, an antioxidant enzyme booster, anti-lipid peroxidation, and anti- hydroperoxides on the treated diabetic rats. This can be attributed to the beneficial bioactivity and antioxidant nature of the extracts.

5. Conclusion

The brain demonstrates a series of morphological and functional alterations during diabetes mellitus which can trigger diseases including Alzheimer's disease, probably as a result of the oxidative stress. The treatment of diabetic rats with polyphenols extracted from *Syzygium cumini* leaves significantly inhibit the activities of AChE and BChE, increased the antioxidant enzymes activities, and decreased the levels of lipid peroxidation and hydroperoxides. This may be attributed to the readily absorbable and antioxidant nature of the polyphenols of *Syzygium cumini* leaves.

Conflict of Interests

The authors declare no conflict of interests.

Ethical Approval

This study was approved by ABUAD Ethical Committee.

Acknowledgment

The authors express their gratitude to all the technologists at the Biochemistry and Chemistry laboratories where the experiment took place.

References

Adam-Vizi V and Seregi A. 1982. Receptor independent stimulatory effect of noradrenaline on Na, K-ATPase in rat brain homogenate. Role of lipid peroxidation. *Biochem Pharmacol.,* **34**: 2231-2236.

Ajiboye BO and Ojo OA. 2014. Effects of aqueous leaf extract of *Senecio biafrae* on hyperglycaemic and haematological parameters of alloxan induced diabetic rats. *PharmacolOnline,* **3**:163 169.

Akanksha A, Srivastava A and Maurya R. 2010. Antihyperglycemic activity of compounds isolated from Indian medicinal plants. *Ind J Exp Biol*., **48**:294-298.

Akinyemi AJ, Oboh G and Ademiluyi AO. 2015. Local salt substitutes "Obu-otoyo" activate acetylcholinesterase and butyrylcholinesterase and induce lipid peroxidation in rat brain. *Interdiscip Toxicol.,* **8(3)**: 139–145.

Bayes JW and Thrope SR. 1999. Role of oxidative stress in diabetic complications. *Diabetes*, **48**:1-9.

Belle NAV, Dalmolin GD, Fonini G, Rubim MA and Rocha JBT. 2004. Polyamines reduces lipid peroxidation induced by different prooxidant agents. *Brain Res.,* **1008**:245-251.

Blockland A. 1995. Acetylcholine: a neurotransmitter for learning and memory? *Brain Res Rev.,* **21**: 285–300.

Carney JM, Strake-Reed PE, Oliver CN, Landum RW, Chang MS, Wu JF and Floyd RA. 1991. Reversal of age-related increase in brain protein oxidation, decrease in enzyme activity and loss in temporal and spatial memory by chronic administration of the spin trapping compound N-tert-buty-alfa-pheynitrone. *PNAS,* **88**:3633-3636.

Chang YT, Chang WN, Tsai NW, Huang CC, Kung CT and Su YJ. 2014. The Roles of biomarkers of oxidative stress and antioxidant in Alzheimer's disease: A Systematic Review. *BioMed Res Inter.,* **2014:** 1-11.

ChuY, Sun J, Wu X and Liu RH. 2002. Antioxidant and antiproliferatve activity of common vegetables. *J Agric Food Chem*., **50 (23**): 6910-6916.

Ellman GL, Courtney KD, Andres V and Featherstone RM. 1961. A new and rapid colorimetric determination of acetylcholinesterase activity. *Biochem Pharmacol*, **7**:88–95.

Gallegos MEH, Zannatha MMI, Osornio EG, Sanches AS and Rio FAP. 2001. Immediate and delayed effects of lead on AChE, GSH-T and thiols in the substantia nigra, neostriatum and cortex of the rat brain. *J Appl Toxicol*.,**21**:397–401.

Gill TS, Teware H and Pande J. 1991. *In vivo* and *in vitro* effects of cadmium on selected enzymes in different organs of the fish *Barbus conchonius* Ham. (Rosy barb). *Comp Biochem Physiol Part C,***100**: 501–505.

Grundman M, Grundman M and Delaney P. 2002. Antioxidant strategies for Alzheimer disease. *Proc Nutr Soc*., **61**: 191-202.

Ingkaninan K, Temkitthawon P, Chuenchon K, Yuyaem T and Thongnoi W. 2003. Screening for acetylcholinesterase inhibitory activity in plants used in Thai traditional rejuvenating and neurotonic remedies. *J Ethnopharmacol.,* **89**, 261–264.

Jiang ZY, Hunt JV and Wolff SP. 1992. Ferrous ion oidationin the presence of xylenol orange for detection of lipid hydroperoxide in low–density lipoprotein. *Anal Biochem,* **202**:384-387.

Jollow DJ, Mitchell JR, Zampaglione N and Gillete JR. 1974. Bromobenzene induced liver necrosis. Protective role of glutathione and evidence for 3,4-bromobenze oxide as a hepatotoxic metabolite. *Pharmacol.,* **22**:151-169.

Kaleem M, Asif M, Ahmed QU and Bano B.2006. Antidiabetic and antioxidant activity of *Annona squamosa* extract in streptozotocin-induced diabetic rats. *Singapore Med J*., **47(8):** 670–675.

Kazeem MI, Akanji MA, Yakubu MT and Ashafa AOT. 2013. Protective effect of free and bound polyphenol extracts from ginger (*Zingiber officinale* Roscoe) on the hepatic antioxidant and some carbohydrate metabolizing enzymes of streptozotocin-induced diabetic rats. *Evidence-Based Complement Alternative Med.,***2013,** article ID 935486.

Khan RA.2012. Effects of *Launaea p rocumbens* on brain antioxidant enzymes and cognitive performance of rat. *BMC Complement Alternative Med.,* **12**:219.

Lane RM, Potkin SG and Enz A.2006. Targeting acetyl-cholinesterase and butyrylcholinesterase in dementia. *Intl. J. Neuropsycopharmacol*, **9**:101-124.

Misra HP and Fridovich I. 1972. The role of superoxide anion in the autoxidation of epinephrine and a simple assay for superoxide dismutase. *J Biol Chem*., **247**:3170-3175.

Orhan I, Sener B, Choudhary MI and Khalid A. 2004. Acetylcholinesterase and butyrylcholinesterase inhibitory activity of some Turkish medicinal plants. *J Ethnopharmacol,* **91**: 57–60.

Pari L and Latha M. 2005. Antidiabetic effect of *Scoparia dulcis*:effect on lipid peroxidation in streptozotocin diabetes. *Gen Physiol Biophys*., **24(1):** 13–26

Pari L and Latha M. 2004. Protective role of *Scoparia dulcis* plant extract on brain antioxidant status and lipid peroxidation in STZ diabetic male Wistar rats. *BMC Complement Alternative Med.,* **4**:16.

Querfurth HW and LaFerla FM. 2006. Alzheimer's disease. *N Engl J Med*. ,**2010**; 362:329-344.

Rotruck JT, Pope AL, Ganther HE, Swanson AB, Hafeman DG and Hoekstra WG. 1973. Selenium: biochemical role as a component of glutathione peroxidase. *Science,***179**:588-590.

Sanches JR, França LM, Chagas VT, Gaspar RS, dos Santos KA, Gonçalves LM, Sloboda DM, Holloway AC, Dutra RP, Carneiro EM, Cappelli APG and Paes AM. 2016. Polyphenol-rich extract of *Syzygium cumini* leaf dually improves peripheral insulin sensitivity and pancreatic islet function in monosodium l-glutamate-induced obese rats. *Frontiers in Pharmacol*., **2016**; 7:48

Schneider LS. 2001. Treatment of Alzheimer's disease with cholinesterase inhibitors. *Clin Geriatr Med.,* **17**:337-358

Sharma J, Chawla R, Kumar R, Sharma A, Sharma RK and Arora R. 2013. *Camellia sinensis* as a Safe Neuroprotective Radiation Counter Measure Agent. *Inter J Pharm Sci Inven.,* **2**: 26-33

Sinha AK. 1972. Colorimetric assay of catalase. *Anal Biochem*.,**47**:389-394.

Soon YY and Tan BKH. 2002. Evaluation of the hypoglycemic and anti-oxidant activities of *Morinda ofcinalis* in streptozotocin-induced diabetic rats. *Singapore Med J.,***43(2):** 77–85.

Taylor P. 1996. Anticholinesterase agents. In**: Goodman & Gilman's the Pharmacological Basis of Therapeutics. (9th Ed).** Hardman JG, Gilman AGand Limbird LE (Eds.). New York: McGraw-Hill Companies, pp 161-176.

Uddin MS, Al Mamun1 A, Hossain AS, Ashaduzzaman M, Noor MAS, Hossain MS, Uddin MJ, Sarker J and Asaduzzaman M.

2016. Neuroprotective effect of *Phyllanthus acidus* L. on learning and memory impairment in scopolamine-induced animal model of dementia and oxidative stress: Natural Wonder for Regulating the Development and Progression of Alzheimer's disease. *Advances in Alzheimer's Dis.,* **5**: 53-72.

Uttara B, Singh AV, Zamboni P and Mahajan RT. 2009. Oxidative stress and neurodegenerative diseases: a review of upstream and downstream antioxidant therapeutic options. *Current Neuropharmacol,* **7**:65-74

Vincent AM, Russell JW, Low P and Feldman EL. 2004. Oxidative stress in the pathogenesis of diabetic neuropathy. *Endocrine Reviews*, **25 (4):** 612–628.

Wacker P, Nunes PV and Forlenza OV. 2005. *Delirium* e demência no idoso: existem fatores de risco comuns? *Ver Psiq Clín*., **32**: 113–118.

Yu BP. 1994. Cellular defense against damage from reactive oxygen species. *Physiol. Rev.* **74**:139-162.

Zar JH. 1984. **Biostatistical Analysis**. Englewood Cliffs, NJ: Prentice Hall.

JJBS

Volume 11, Number 2,June 2018
ISSN 1995-6673
Pages 171 - 177

Jordan Journal of Biological Sciences

An Evaluation of Contaminant Body Burdens in Selected Fish Species: Associating Toxicity to Upgrade the Hazard Assessment

Victor E. Okpashi*, Victor N. Ogugua, Chibuike S. Ubani and Nwamaka O. Juliet

.

Department of Biochemistry, University of Nigeria, Nsukka, Nigeria

Received August 16, 2017; Revised November 3, 2017; Accepted November 14, 2017

Abstract

The health problems that are related to chronic exposures to polycyclic aromatic hydrocarbons (PAHs) include decreased immunity, Cataracts, kidney and liver damage, breathing problems, asthma or (lung- function abnormalities) skin redness and inflammation. Induced by a repeated contact with PAHs were found to cause various forms of cancer in aquatic animal models, especially a form that can easily integrate with the cell and corrupt the DNA. This study presents an assessment of the recalcitrant body residues of some legacy contaminants in selected fresh fish species. Three different species of fish, namely the African Red snapper (*Lutjanus agennes*), the Great barracuda (*Sphyraena barracuda*), and the African cat- fish (*Clarias gariepinus*), were sampled in a 1-km stretch of the Qua Ibeo River, Nigeria. The specimens were screened for PAHs using gas chromatography-mass spectroscopy. This study revealed the sixteen priority PAHs, listed by the United State Environmental Protection Agency (USEPA) as carcinogenic. The bio-concentration factor was calculated as the ratio of the concentration of particular PAHs in the tissues to its water-free PAHs concentration. Results showed that the PAHs concentration is beyond the permissible limit. Considering these results, one can infer that the Qua Iboe River and the biota are contaminated by PAH, with the risks of bio-accumulation posing threats to human health through the consumption of fish and aquatic organism foods.

Keywords: PAHs, Fish, Body Burden, Risk Assessment, Toxicity, Aquatic Pollution

1. Introduction

On the whole, most scientific reports and researches focus on the ability to measure the body burden of chemicals in biota. Although the bioaccumulation of a contaminant is an important issue, bioaccumulation in and of itself is not a hazard. The critical question is: at what point does bioaccumulation result in body burdens that lead to adverse effects on individual organisms (prey and predator species) and ultimately the whole ecosystems (Meador *et al.,* 1995). One increasingly popular approach linking body burdens of individual organisms to toxicological effects in that organism is the critical body residue (CBR). This approach shifts the focus from quantifying concentrations in water to predicting toxicity by measuring concentrations in tissues. The CBR approach has several advantages over commonplace approaches that measure concentrations in water and sediments, as discussed in McCarty and MacKay (1993). The body burden of polycyclic aromatic hydrocarbons (PAHs) is determined by the balance between uptake and elimination, where each can be influenced by many factors. The determination of uptake and elimination may suggest measuring tissue concentrations at two different times through routine monitoring. The rate at which these processes occur is more instructive, and may be used to compare species differences, and predict steady-state accumulation. Elimination can be accomplished by passive diffusion when the external concentrations are lower than the internal concentrations favoring an outward flux and enzymatic pathways that convert the hydrophobic parent compounds to more polar metabolites that are readily excreted by some taxa with kidneys or kidney-like organs (such as annelids, molluscs and arthropods) (Hom *et al*., 1995). The conversion of the hydrophobic PAHs to a polar metabolite decreases its ability to diffuse through the gill membrane, favoring the excretory route. The rate of elimination may be affected by environmental factors such as temperature, salinity, physiological factors, reproductive state, age, sex, stress, enzyme-induction, route of uptake, chemical hydrophobicity and exposure history (Mckim, 1994). An uptake rate constant describes the fractional changes in water concentration per unit time (hr.-1) and depends on relative sizes of the fish and water compartments (Barron *et al.,* 1990; Landrum *et al.,* 1992). For those organic contaminants that are metabolized, the uptake and the potential for accumulation are more difficult to determine, although the quantification of metabolites will greatly improve the assessment. Even at

* Corresponding author. e-mail: vic2reshu@gmail.com.

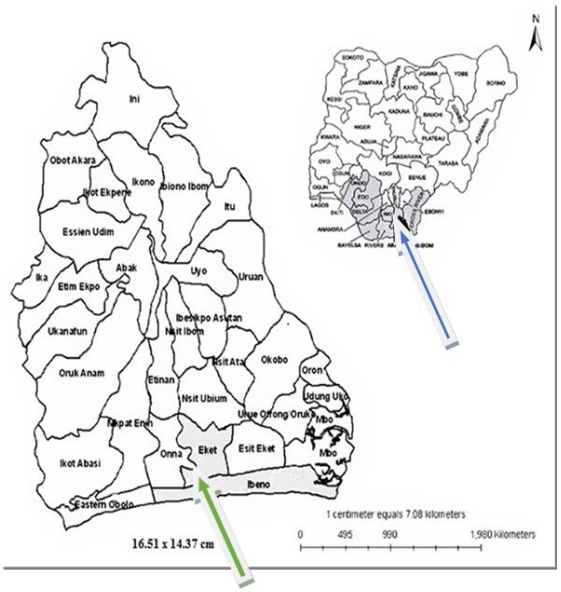
extremely-low environmental concentrations of PAHs (i.e., sometimes below detection limit by analytical instruments), aquatic organisms will accumulate PAHs because partitioning to the organism is thermodynamically favored, and the bio-concentration factors are quite high - (generally in the range of $l0^2$ - $l0^6$) - (Connell, 1991). The pattern of synergism in PAHs uptake, in the presence of other contaminants, has been rarely studied. One study found inhibition in the uptake of naphthalene by Eastern oyster (*Crassostrea virginica*) when presented with PCBs and benzo[a]pyrene (BaP) (Fortner and Sick, 1985). When the oysters were exposed to seawater with only naphthalene, uptake of both dissolved and particulate naphthalene produced tissue burdens of this compound that were always slightly higher.

Conversely, the authors concluded that naphthalene and PCBs had no effect on BaP uptake. In contrast, Stein *et al.* (1984) observed higher body burdens of BaP metabolites in the English sole (*Parophrys vetulus*) exposed to sediment containing BaP and PCBs than in fish exposed to sediment containing only BaP. This may occur because enzyme-induction led to a greater metabolism and higher amounts of metabolites that were not excreted. In the pelagic environment, fish and invertebrates may take up PAHs from water through diffusion across their gills and integuments and via their diet (Knezovich *et al*., 1987). Fish and invertebrates associated with sediment, either living in the substrate (infaunal) or on the surface of sediments (epibenthic) may accumulate PAHs via the diffusion of waterborne PAHs across their gills and integuments, and by dietary uptake. The contribution of PAHs through water and dietary sources to tissue burdens was examined by modeling. A series of curves showing the percentage uptake from water as a function of hydrophobicity was developed for two different modes of feeding (Hom *et al*., 1995). Generally, the metabolism of parent compounds is the principal confounding factor in assessing the exposure to PAHs. Therefore, the - measurement of parent hydrocarbons in any organism that is actively metabolizing PAHs may yield partial information on the total PAHs-derived body burden. Consequently, a direct measurement of parent hydrocarbon would be a useful quantitative parameter in animals that have minimal capacity for PAHs metabolism (Barron *et al*., 1990). Neff *et al.* (1976) reported that the rate of the release of a PAH is dependent on molecular weight and presumably hydrophobicity, with higher molecular weight compounds being released much slowly than low molecular weight compounds. Parent PAHs and their unbound metabolites are not highly persistent in fish, and their residual levels remaining in tissues and fluids two weeks after the exposure are generally minimal (Varanasi *et al*., 1989). However, reactive PAHs metabolites that covalently bind to DNA are quite persistent in fish. In studies with PAHs and nitrogen-containing aromatics, Stein *et al.* (1993), showed that BaP, which is a model mutagenic PAH, and 7H-dibenzo- [c,g]carbazole; a model nitrogen containing mutagenic PAH, form highly persistent DNA adducts in fish. The bio-concentration factor (BCF) is the ratio of the concentration of a particular chemical in the tissue to its concentration in the water. It should be kept in mind that the BCF is relevant for the accumulation from water. Therefore, in order to compare BCFs among different biota, it is important to confirm that water is the only route of uptake (Macarty, 1986).

2. Materials and Methods

2.1. Research Area

Eket is the second largest city in Akwa Ibom State in Nigeria. The name also refers to the indigenous ethnic group of the region and their language. The area of Eket's local government occupies the South central portion of Akwa Ibom State. It lies entirely in the tropics with territorial expense, spanning Northwards between Latitude 4° 33″ and 4° 45″ and Eastward between Longitude 7° 52″ and 5° 02″. Eket is bounded on the north by Nsit Ubium LGA, on its east by Esit Eket LGA, on the west by Onna LGA, and on the south by Ibeno LGA/Bright of Bonny Figure 1. At present, there are activities involving oil exploitation from Shell and Exxon Mobil, a thriving hub of a new oil and gas business, with more than 250 companies providing support services such as catering, flights and exports. An oil refinery is currently under construction in the outskirts of the city along the Oron road.



Key: The green arrow point to Map of Eket

Figure 1. Geography of the Project location showing the map of Nigeria and Akwa Ibom State

2.2. Description of the Study Sites

The study was conducted in the Qua Ibeo River (aka Kwa Ibo River), that rises near Umuahia in Abia State in Nigeria, and flows in a southeastern direction through Akwa Ibom State to the Atlantic ocean. The river feeds a zone of mangrove swamps linked by creeks and lagoons separated from the sea by a low and narrow ridge of sand. Ibeno, on the eastern side of the River ≈ 3 km from the river mouth, is one of the largest fishing settlements on the Nigerian coast. Four sampling stations were established, 1km apart, in the upper and middle reaches of the River based on ecological settings and the intensity of human activities. At each station, three sampling sites were selected, making a total of twelve sample points for the present study.

Station 1: This station was located in the upper reaches of the river at Ikot Ikpe and Ikot Akpoenang at latitude 4 - 55.8" and longitude 7 - 40.8". The main socio-economic activities in this area are mainly fishing and boat-building along the river banks. The water quality is relatively good at this location based on visual evaluation.

Station 2: This location receives effluents from a slaughter house located along the Ikot Aroku and Ikot Naidiba village road. There is also discharge of domestic and municipal waste into the river. The main socio-economic activities in this area include sand-mining for both domestic- and commercial purposes. It is about 1 km downstream from Station 1, straddling latitude 4 - 22.9", and longitude 7 - 13.8".

Station 3: This site is located along Eket-Etinan road, within Ebiyan and Ndon directly opposite the Onna local government area. Socio-economic activities at this location include washing of cars and motor bikes, laundry and bathing. The vegetation is dominated by bamboo trees. The site lies about 1km downstream of Station 2, straddling latitude 4 - 23.2"and longitude 7- 40.2".

Station 4: This is located at Ndilla. The opposite villages across the river are Odio and Ale Ebukuku in the lower reaches of the river near the estuary. This descended into Ibeno local government area, see Figure 2. The socio-economic activities include fishing and sand mining. The vegetation is mainly mangrove forests. The area is turbid due to sediments from sand mining as well as the impacts of socio-economic activities upstream. This site is also very deep due to the mining activities which excavate the river bed. It is located 1 km downstream of Station 3 straddling latitude 4- 43.5" and longitude 7 - 53.3".

Figure 2. Map of Eket local government area of Akwa Ibom State, Nigeria

2.3. Sample Collection and Analysis

The river water samples were collected using twelve amber bottles, each 100mL from twelve different points in Eket area of Akwa Ibom state on the second of September, 2015 between 10 a.m and 3 p.m along the coast. The samples collected were extracted with n-hexane before concentrating the analytes or target compounds within thirty minutes.

Fish sampling was randomly conducted to ensure that the representative samples of biota were included in the samples for analysis. Some variables that influenced the site selection based on field work included the proximity to oil-well locations, gas flaring from Bonny Bright, high population density, socio-economic activities, particularly fishing within the area, with heavy presence of sewage, indiscriminate disposal of solid waste in and around the shore of Qua-Iboe river. Locally-consumed fresh Yellow tail (*Seriola lalandi*), Atlantic Croker (*Micropogonias undulates*), and Tilpia (*Oreochromis niloticus*) were collected for this study by a resident fisherman using set nets. These species of fishes, irrespective of their sex and age were weighed. The fishes were wrapped in hexane-rinsed aluminum foil. They were labelled and placed inside closed-glass vessels containing ice pack, and were kept at below -20°C before being taken for laboratory analysis.

2.4. Sample Preparation, Extraction and Clean-Up Procedure of Fish Samples for Analysis

Prior to extraction, the fish samples were descaled using knife, and subsequently dissected to obtain the tissues. From each specimen, an excision of 15 g of the fish tissue was placed in a clean mortar, and ground with pestle with 40g of anhydrous sodium sulphate - until the sample was completely dried and homogenized. The sample extraction was carried out using dichloromethane (DCM). A sub-sample of 10g of the homogenized sample was placed in 50ml extraction bottle and 1mL of 60ng/mL of 1- Chloro-octadecane surrogate standard was added in the extraction bottle. The content was agitated or vortexed for five hours, and was allowed to settle for one hour. The sample was then carefully filtered through a funnel fitted with cotton wool, silica gel, and Sodium Sulphate (Na_2SO_4) in a clean volumetric flask. The residue was further washed and made-up to volume using the extracting solvent. The sample was concentrated to 2 ml for PAHs analysis using a gas Chromatography Tandem-Mass Spectroscopy (GC-MS).

2.5. Extraction of Fish and Water Samples for PAH Determination

The liquid-liquid extraction procedure was used in this analysis as follows. One litre of the sample was extracted in a 2L glass separator funnel fitted with a glass stopper using 30 mL of hexane as extract. The separator funnel was vigorously shaken for three minutes, and the organic layer was allowed separating clearly from the aqueous phase for a minimum of five minutes, and - the organic layer was then collected into a separate glass bottle. The extraction was repeated thrice for each sample. Water residues were expelled from the organic layer by passing extracts through funnels containing anhydrous Sodium Sulphate. Extracts were concentrated using rotary evaporators with the water bath preset at 85^0 C. Concentrated extracts were transferred to a pre-weighed sample bottle and were evaporated to dryness.

2.6. Calculation of Bio -Concentration Factor (BCF)

The bio-concentration factor was investigated using the McCarty (1986) method. The bio-concentration factor (BCF) is the ratio of the concentration of a particular chemical in the tissue to its concentration in water

(equation 1) below. Considering that - BCF is relevant only for the accumulation from water to compare with other BCFs, it is important to confirm that water is the only route of uptake. Conversely, the bioaccumulation factor (BAF) which is generally computed as the ratio between the contaminant concentrations in the tissues and multiple external sources (e.g., sediment, water, and diet) is useful in determining the tendency of hydrophobic compounds to accumulate in the tissue.

$$BCF\ with\ free\ PAHs\ in\ water = \frac{Tissue}{Water\ free} \quad Equation\ 1$$

BCF, (bio-concentration factor with free PAH in water) = [Tissue]/ [Water free]

BCF is BCF predicted. For example, the equation from McCarty (1986); = 0.046 KOw.

2.7. Statistical Analysis

All investigations were carried out in triplicate, and the data obtained were presented as mean ± standard deviation using descriptive statistics. The one-way analysis was used to compare mean variance among the samples. Significance was accepted at $p < 0.05$ level using SPSS software, (version 18).

3. Results

3.1. Bioaccumulated PAHs in African Red Snapper (Lutjanus agennes) Fish

Table 1 shows the mean concentration of individual PAHs in river-water samples, mean bio-concentration of individual PAHs in the fish tissue, and the bioaccumulation factor of individual PAHs in *L. agennes* specimen. Extrapolated data revealed that *L. agennes* had the highest PAHs body load compared with the other fish species analyzed. For instance, the BCF for BaP was 2.15±6.19, while indeno (1, 2, 3, cd) pyrene was the highest body load or burden at 2.19±1.13. Meanwhile, other PAHs compounds recorded higher BCF values beyond the permissible limit. For example, naphthalene had 1.27±8.78, 2-methylnaphthalene at 1.07±0.23, acenaphthylene at 1.81±0.36, acenaphthene at 1.09±0.04, fluorene at 1.03±0.18, phenanthrene at 1.07±0.17 and anthracene 1.07±2.11, respectively.

Table 1. Bio-accumulated PAHs in African Red snapper (*Lutjanus agennes*) fish.

Polycyclic aromatic hydrocarbons (PAHs)	Mean ± SD of Contaminant in African Red snapper fish (ppm)	Mean ± SD Bio-concentration factor (BCF)
Naphthalene (ppm)	29.763±1.001	1.277±8.780*
2-Methylnaphthalene (ppm)	30.260±0.060	1.072±0.231
Acenaphthylene (ppm)	30.100±0.081	1.817±0.368
Acenaphthene (ppm)	30.133±0.049	1.092±0.040
Fluorene (ppm)	28.106±0.023	1.031±0.187
Phenanthrene (ppm)	30.533±0.367	1.074±0.174
Anthracene (ppm)	30.360±0.467	1.074±2.113*
Fluoranthene (ppm)	27.416±5.035	0.997±12.556*
Pyrene (ppm)	28.773±1.157	1.020±1.060*
Benzo (a) anthracene (ppm)	21.480±2.295	0.817±10.432*
Triphenylene (ppm)	30.183±0.119	1.038±0.361
Benzo (e) pyrene (ppm)	26.623±6.160	0.965±29.194*
Benzo (a) pyrene (ppm)	29.773±0.687	2.146±6.189*
Indeno (1,2,3,cd) pyrene (ppm)	30.116±0.241	2.186±1.131*
Benzo (g,h,i) perylene (ppm)	30.536±0.730	1.075±3.476*
Dibenzo (a,h) anthracene (ppm)	30.526±0.180	1.078±1.782*
000053-70-3-benzo(e) pyrene ppm)	29.490±1.625	1.059±9.503*

Results are mean ± SD for 3 determinations

Column marked with asterisk (*) indicates standard deviation greater than the mean. This is because the water free PAHs were considerably higher than the partitioned PAHs in fish tissue after computation.

3.2. Bio-Accumulated PAHs in Great Barracuda (Sphyraena barracuda) Fish

Table 2 shows the mean concentration of individual PAHs in river water samples, mean bio-concentration of individual PAHs in the fish tissue, and the bioaccumulation factor of individual PAHs in the Great Barracuda (*Sphyraena barracuda*) fish. The bio-concentration values of PAHs in the fish tissue indicated a low PAH contamination in all the fish samples analyzed. The BCF values are considerably lower compared to other fish species. For example, BeP had 0.361±1.602, BaP 0.683±6.099 and Indeno (1, 2, 3, cd) pyrene at 0.689±1.709. Other PAHs compounds such as 2-methylnaphthalene, acenaphthylene, acenaphthene, fluorine, phenanthrene, anthracene, fluoranthene and pyrene were recorded in lower BCFs, respectively.

Table 2. Bio-accumulated PAHs in the Great Barracuda (*Sphyraena barracuda*) fish.

Polycyclic aromatic hydrocarbons (PAHs)	Mean ± SD of Contaminant in Barracuda fish (ppm)	Mean ± SD Bio-concentration factor (BCF)
Naphthalene (ppm)	9.360±0.795	0.402±6.974*
2-Methylnaphthalene (ppm)	9.550±0.446	0.339±1.930*
Acenaphthylene (ppm)	9.4300±0.578	0.569±2.627*
Acenaphthene (ppm)	9.806±0.596	0.355±0.492
Fluorene (ppm)	8.777±1.154	0.322±9.382*
Phenanthrene (ppm)	8.726±0.903	0.307±0.43
Anthracene (ppm)	10.086±0.030	0.357±0.135
Fluoranthene (ppm)	9.766±0.516	0.355±1.286*
Pyrene (ppm)	9.753±0.575	0.346±0.527
Benzo (a) anthracene (ppm)	9.913±0.375	0.377±1.705*
Triphenylene (ppm)	9.826±0.422	0.337±1.283*
Benzo (e) pyrene (ppm)	9.960±0.338	0.361±1.602*
Benzo (a) pyrene (ppm)	9.473±0.677	0.683±6.099*
Indeno (1,2,3,cd) pyrene (ppm)	9.490±0.364	0.689±1.709*
Benzo (g,h,i) perylene (ppm)	9.800±0.616	0.345±2.933*
Dibenzo (a,h) anthracene (ppm)	10.093±0.565	0.357±5.594*
000053-70-3-benzo(e) pyrene ppm)	9.676±0.480	0.348±2.807*

Results are mean ± SD for 3 determinations

Column marked with asterisk (*) indicates standard deviation greater than the mean. This is because the water free PAHs were considerably higher than the partitioned PAHs in fish tissue after computation.

3.3. Bioaccumulated PAHs in the African Cat- Fish (Clarias gariepinus)

Table 3 shows the mean concentration of individual PAHs in river water samples, mean bio-concentration of individual PAHs in the fish tissue, and the bioaccumulation factor of individual PAHs in *Clarias gariepinus* samples. Results followed a similar trend to table 1. Though there is dissimilarity in values in terms of concentration and the bio-concentration factor. Considering PAH body load or burden in cat fish, results showed that the PAHs compounds accumulated were tolerable. For instance, Benzo (a) anthracene recorded 0.365±2.722 ppm, and Benzo (e) pyrene was 0.367±0.286 ppm. These values are below the 0.5 ppm threshold given by McCarthy *et al*., (1986) as the toxic level in aquatic organisms.

Table 3. Bio-accumulated PAHs in cat-fish *(Clarias gariepinus).*

Polycyclic aromatic hydrocarbons (PAHs)	Mean ± SD of Contaminant in cat - fish (ppm)	Mean ± SD Bio-concentration factor (BCF) ppm
Naphthalene (ppm)	10.517±0.453	0.451±3.974*
2-Methylnaphthalene (ppm)	10.476±0.551	0.371±2.385*
Acenaphthylene (ppm)	10.170±0.500	0.613±2.272*
Acenaphthene (ppm)	10.097±0.023	0.366±0.019
Fluorene (ppm)	10.146±0.115	0.372±0.934
Phenanthrene (ppm)	10.400±0.294	0.366±0.140
Anthracene (ppm)	10.373±0.458	0.367±2.072*
Fluoranthene (ppm)	11.646±0.803	0.423±2.002*
Pyrene (ppm)	10.360±0.312	0.367±0.286
Benzo (a) anthracene (ppm)	10.613±0.512	0.404±2.327*
Triphenylene (ppm)	10.206±0.215	0.351±0.653
Benzo (e) pyrene (ppm)	10.323±0.195	0.374±0.924
Benzo (a) pyrene (ppm)	9.436±1.411	0.680±12.711*
Indeno (1,2,3,cd) pyrene (ppm)	9.656±1.064	0.701±4.995*
Benzo (g,h,i) perylene (ppm)	10.133±1.021	0.357±4.862*
Dibenzo (a,h) anthracene (ppm)	10.336±0.275	0.365±2.722*
000053-70-3-benzo(e) pyrene ppm)	10.193±0.020	0.366±0.117

Results are mean ± SD for 3 determinations

Column marked with asterisk (*) indicates standard deviation greater than the mean. This is because the water free PAHs were considerably higher than the partitioned PAHs in fish tissue after computation.

4. Discussion

Studies on wildlife have revealed some modification of the gonadal, reproductive development and hormones (Cynthia *et al.,* 2004). A number of abnormalities in the reproductive system of various wildlife species correlate with abnormalities found in human population. These substances in our environment, food, and consumer products interfere with hormone biosynthesis and metabolism resulting in a deviation from normal homeostatic control or reproduction. Reports from aquatic animal models, human clinical observations, and epidemiological studies show that endocrine disrupting compounds are a significant concern for public health. The available field evidence gives proof to the ubiquitous nature of PAHs in environmental media and fish.

The exposure to Benzo (a) pyrene may damage the reproductive system and cause cancer. Ingestion of Benzo (e) pyrene may cause gastrointestinal irritations. Dermal contact with Benzo (a) pyrene may lead to skin irritation. In the natural environment, Benzo (a) pyrene occurs as part of a mixture of polycyclic aromatic hydrocarbons (PAHs). The full effects of Benzo (a) pyrene on human health are unknown, however studies have shown that inhalation of PAHs or dermal contact with PAHs for extended periods of time can cause cancer. The maximum permissible concentrations (MPCs), the serious risk concentrations (SRC) for ecosystems, and the sixteen known polycyclic aromatic hydrocarbons (PAHs) were derived by Verbruggen (2012). This was computed for all individual PAHs in water and fish. Bioavailability and organism's physiology are the two important variables with major effects on chemical contaminant body burdens. Considering the wide spread of environmental PAHs, only the bioavailable fraction can enter the organism. Results revealed different mean PAHs bio-accumulation in the African Red snapper (*Lutjanus agennes*). These bio-accumulated toxicants in the fish are beyond the USEPA permissible limits - and the recommendation limit postulated by McCarty (1986) of = 0.046- KOw. Physiological factors, including lipid levels, the rates of uptake and elimination i.e. (metabolism, diffusion, and excretion), determine the contaminant body burden (Landrum *et al.,* 1994). Uptake is an important factor when determining body burden, since this is controlled by factors associated with bioavailability and organismal physiology. It is believed that the process of uptake of hydrophobic compounds is passive (vis-a-vis active transport) and is controlled by diffusion pressure (fugacity) because of the difference between the environmental matrix and the tissue load (Mckim, 1994). The uptakes of PAHs from water, and factors that control the concentration of the free PAHs (non-sorbed) are important, and can be attributable to toxicity and risk.

Comparatively, the Great Barracuda fish (*Sphyraena barracuda)* bio-accumulated lesser contaminants than the African Red Snapper fish. For this model, Benzo (a) pyrene 0.683±6.099 ppm and Indeno (1, 2, 3, cd) pyrene 0.689±1.709 ppm had the highest concentration, and posed a bigger risk burden on the organism. This study presents evidence that differential ability of fish species and tissues to bio-transform PAHs to more water-soluble metabolites and metabolites half-life may result in differences in body burden. Some studies indicate that a fraction of persistent PAH remains in the tissues of organisms that are exposed to PAHs for long periods, even in species capable of metabolizing these xenobiotics. Results of PAHs bio-accumulation indicate a higher degree and variability of PAHs body burdens in *C.gariepinus*. Comparing the toxicant body burdens in these aquatic fish species showed that they differ from other species, although they were all fished from the same river. Several factors may be attributed to the variation in concentration. Understanding the mechanism of chronic exposure to PAHs by fish is desirable when evaluating the population of aquatic animal models and the possibility of transferring contaminant body burdens in fish to humans via the food chain. Additionally, the carcinogenicity of PAHs presents a human health risk because some PAHs are bio-transformed to reactive metabolites which interact with DNA adducts. The ability to predict tissue burdens in aquatic organisms is important in the assessment of hazard and toxic effects. Considering the lethal body burdens of non-ionic hydrophobic compounds such as parent PAHs compounds, acting by narcosis, they are believed to be in the range of 2-6 pmol/g (wet wt) (McCarty 1990). For species with no capacity to metabolize PAHs, assessing contaminant body burdens in light of this synergetic reactions will be beneficial in determining deleterious effects. Perhaps, these body burdens can be applicable in the relationships between chemical exposure and ecological effects on aquatic species.

5. Conclusion

This study evaluated contaminant body load from existing bioaccumulation, using model organisms as screening tools. The use of indigenous fish species was desirable since the results of these species would have greater ecological significance to predict perceived risks or hazards associated with aquatic pollution. The direct measurement of bio-concentration gives an insight into toxicant tissue residues at the time and location of the sampling. Direct measurement was accomplished by measuring tissue concentrations in the field-collected organisms. It is concluded that the toxicant body burdens in the assessed fish species were beyond the permissible limits as described by USEPA.

References

Barron MG, Stehly GRand Hayton WL. 1990. Pharmacokinetic modeling in aquatic animals. I. Models and concepts. *Aquatic Toxic* **17**: 187-212.

Connell DW. 1991. Extrapolating test results on bioaccumulation between organism groups. *In*: **Bioaccumulation in Aquatic Systems-Contributions to the Assessment**, R. Nagel R and R. Loskill R. (Eds) , Proceedings of an international workshop, Berlin, 1990. VCH Publishers, Weinheim, pp 133-149.

Cynthia A, Heinlein R and Chawnshang C. 2004. Androgen receptor in prostate cancer. *Endocrine Revi.,***25**(2):276–308.

Fortner AR and Sick LV. 1985. Simultaneous accumulations of naphthalene, a PCB mixture, and benzo(a)pyrene, by the oyster, *Crassostrea virginica*. *Bull Environ Contam Toxicol*. **34**:256-264.

Hom T. Varanasi U. Stein JE. Sloan CA. Tilbury, KL and Chan SL. 1995. Assessment of the exposure of subsistence fish to aromatic compounds following the Exxon Valdez oil spill. In: **Exxon Valdez Symposium Proceedings**, Rice SD, Spies RB, Wolfe DA and Wright BA (Eds). American Fisheries Society, Washington, DC.

Knezovich JP. Harrison FLand Wilhelm RG 1987. The bioavailability of sediment-sorbed organic chemicals: a review. *Water Air Soil Pollut* **32**:233-245.

Landrum PF. Dupuis WS Kukkonen J., 1994. Toxicokinetics and toxicity of sediment-associated pyrene and phenanthrene in *Diporeia* spp.: examination of equilibrium-partitioning theory and residue-based effects for assessing hazard. *Environ Toxicol Chem.,* **13**: 1769-1780.

Landrum PF. Lee H Lydy MJ. 1992. Toxicokinetics in aquatic systems: model comparisons and use in hazard assessment. *Environ Toxicol Chem***., 1** (1): 1709-1725.

McCarty LS. 1986. The relationship between aquatic toxicity QSARS and bioconcentration for some organic chemicals. *Environ Toxic Chem.,* **5**: 1071- 1080.

McCarty LS. 1990. A kinetics-based analysis of quantitative structure-activity relationships in aquatic toxicity and bioconcentration bioassays with organic chemicals. Ph.D. thesis, University of Waterloo, Waterloo, Ontario, Canada.

McCarty LS and MacKay D. 1993. Enhancing ecotoxicological modeling and assessment: Body residues and modes of toxic action. *Environ. Sci. Technol*. **27:**1719-1728.

McKim JM. 1994. Physiological and biochemical mechanisms that regulate the accumulation and toxicity of environmental chemicals in fish. *In*: **Bioavailability: Physical, Chemical, and Biological Interactions**, Hamelink JL. Landrum PF. Bergman HL and Benson WH (Eds). Lewis Publishers, Boca Raton, FL, pp 179-201.

Meador JP. Casillas E. Sloan CA Varanasi U. 1995. Comparative bioaccumulation of polycyclic aromatic hydrocarbons from sediment by two infaunal organisms. *Mar Ecol Prog Ser*., **123**: 107-124.

Neff JM. 1976. Polycyclic aromatic hydrocarbons. *In*: **Fundamentals of Aquatic Toxicology,** Rand GM and Petrocelli SR (Eds). Hemisphere Publishing Co., New York, pp 416-454.

Stein JE, Hom T, and Varanasi U. 1984. Simultaneous exposure of English sole (*Parophrys vetulus*) to sediment-associated xenobiotics: Part 1 -uptake and disposition of '4C-polychlorinated biphenyls and 3~-benzo[a]pyrene. *Mar Environ Res.,* **13**:97-119.

Stein JE, Reichert WL, French B and Varanasi U. 1993. 32P-postlabeling analysis of DNA adduct formation and persistence in English sole (Pleuronectes vetulus) exposed to benzo[a]pyrene and 7H-dibenzo[c,g]carbazole. *Chem Biol Interact*., **88(1):**55-69.

Varanasi U, Stein JE and Nishimoto M. 1989. Biotransformation and disposition of polycyclic aromatic hydrocarbons (PAH) in fish. *In*: **Metabolism of Polycyclic Aromatic Hydrocarbons in the Aquatic Environment,** Varanasi U (Ed). CRC Press, Boca Raton, FL, pp 94-149.

Verbruggen EMJ. 2012. Environmental risk limits for polycyclic aromatic hydrocarbons (PAHs) for direct aquatic, benthic, and terrestrial Toxicity. National Institute of Public Health and the Environment. RIVM report *607711007/2012*; pp: 165-182.

JJBS

Volume 11, Number 2,June 2018
ISSN 1995-6673
Pages 179 - 186

Jordan Journal of Biological Sciences

Population Parameters of the Minor carp *Labeo bata* (Hamilton, 1822) in the Ganges River of Northwestern Bangladesh

Md. Alomgir Hossen[1], Md. Yeamin Hossain[1,*], Dalia Khatun[1], Fairuz Nawer[1], Most Farida Parvin[1], Ayesha Arabi[2] and Md. Abul Bashar[3]

[1]*Department of Fisheries, Faculty of Agriculture, University of Rajshahi, Rajshahi 6205, Bangladesh*

[2]*Milton Keynes, MK2 2LN, United Kingdom;* [3]*Bangladesh Fisheries Research Institute, Riverine Station, Chandpur, Bangladesh*

Received September 21, 2017; Revised November 5, 2017; Accepted November 17, 2017

Abstract

The Minor carp, *Labeo bata* (Cyprinidae), is one of the important fish species in northwestern (NW) Bangladesh. This study illustrates the population parameters of *L. bata* i.e., population structure (length-frequency distributions; LFDs), growth (length-weight relationships, LWRs; length-length relationships, LLRs), condition factors (allometric, K_A; Fulton's, K_F; relative, K_R; relative weight, W_R), form factor ($a_{3.0}$), reproduction (length at first maturity, L_m) and natural mortality (M_w) in the Ganges River, NW Bangladesh. Samples were collected occasionally between July, 2013 to June, 2014 using different fishing gears i.e., gill net (mesh sizes: 2.5–3.5 cm) and cast net (mesh sizes: 1.5–3.0 cm). A total 157 individuals of *L. bata* were collected, where sixty-nine were male, and eighty-eight were female. The sex ratio did not fluctuate significantly from the anticipated 1:1 ratio (df =1, χ^2=2.30, p>0.05). Total length varied from 7.9 to 25.2 cm and body weight ranged from 4.68 to 181.35g. All LWRs were highly significant (p<0.01), with all r^2 values ≥0.984. The analysis of covariance (ANCOVA) revealed no extensive differences in LWRs between genders (p>0.05). The allometric coefficient (b) indicated positive allometric growth in both sexes (b>3.00, p<0.01). All LLRs were highly correlated (p<0.001), and most of the r^2 values were ≥0.992. Among four condition factors, K_F was the best fitted, and can be used as indicator of safe life for *L. bata* in the Ganges River. Wilkoxon Sign Ranked Test for W_R showed no considerable dissimilarity from 100 for male (p=0.295) and female (p=0.057). The a_{30} for both sexes was 0.0108. The L_m for male and female population was 14.12 cm and 14.60 cm in TL, respectively. The M_w for the population of *L. bata* was 0.86 year^{-1} in the Ganges River. This result would be effective for a sustainable management of this Minor carp in Bangladesh and its adjoining ecosystems.

Keywords: *Labeo bata*, growth, condition, maturity, mortality, Ganges River.

1. Introduction

The Minor carp, *Labeo bata* (Hamilton, 1822), is an important native target species for fishers, much sought-after in Bangladesh because of its high commercial value. It is a freshwater benthopelagic, potamodromous cyprinid, commonly known as Bata in Bangladesh, Bhagan in India, and Bata labeo in Nepal (Froese and Pauly, 2016). It can be found throughout the Indian sub-continent, including Bangladesh, India, Myanmar, Nepal and Pakistan (Robins *et al*., 1991), inhabiting rivers, canals, *haors*, ponds and ditches. In spite of some local threats impacting this species, there is no present information on its population decline. Nowadays, this fish is being cultured commercially, and no risk is expected in the near future. Hence, at the present this species is assessed as a least concern, although it had been assessed as endangered earlier in Bangladesh (IUCN Bangladesh, 2015).

Information regarding the length, weight, sex, first maturity and mortality is of elemental significance to the effective management of an exploitable fish population in that they can be employed to determine a maximum sustainable yield (Chen and Paloheimo, 1994; King, 1997). Nevertheless, several authors reported on the biology, ecology (Talwar and Jhingran, 1991), breeding performance, growth, survival rate, morphometric relationships and the condition factor (Siddique *et al*., 1976; Naeem *et al*., 2012; Hossen *et al*., 2015, Hossain *et al*., 2016a) for this species, but in spite of the economic importance of this species, no prior research was performed on the population parameters of *L. bata*.

Hence, the aim of this research is to elucidate the population parameters including growth pattern (length-

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

weight relationships, LWRs; length-length relationships, LLRs), condition factors (allometric, K_A; Fulton's, K_F; relative, K_R; and relative weight, W_R), reproduction (size at first maturity, L_m), and natural mortality (M_W) of *L. bata* from the Ganges River in northwestern (NW) Bangladesh using a number of specimens with various sizes over a study period of one year.

2. Material and Methods

2.1. Study Area and Sampling

A sum of 157 individuals of *L. bata* was collected occasionally from the Ganges River (known as Padma River in Bangladesh) (Lat. 24.35′ N; Long. 88.64′ E) NW Bangladesh) between July, 2013 to June, 2014 from artisanal fishers. The samples were caught using gill net (mesh sizes 2.5–3.5 cm), and cast net (mesh sizes 1.5–3.0 cm). Fresh samples were instantly chilled in ice on site and were transported to the laboratory and were fixed with 10% buffered formalin for laboratory analysis.

2.2. Fish Measurement

Morphological traits (Total length, TL; fork length, FL; and standard length, SL) were assessed via digital slide calipers (±0.01 cm). The whole body weight (BW) was taken using an electronic balance with a 0.01 g accuracy.

2.3. Sex Ratio and Length-Frequency Distributions (*LFDs*)

Sex determination of samples was done by a microscopic observation of the gonads. The chi-square test was conducted to verify the sex-ratio variance from the anticipated value of 1:1 (male: female). The LFDs were constructed separately for male and female using 1 cm intervals of TL.

2.4. Growth Pattern

The growth pattern was estimated through LWR with the equation: $BW= a*L^b$, where BW is the total body weight (g), and L is the length (TL, FL and SL in cm). Through linear regression analysis, the parameters *a* and *b* were estimated based on natural logarithms: ln (*W*) = ln (*a*) + *b* ln(*L*). Outliers were excluded from the regression analysis by Froese (2006). The *t*-test was employed to confirm whether the *b* values obtained in the linear regressions were significantly departed from the isometric value (*b*=3) (Sokal and Rohlf, 1987). Furthermore, linear regression was applied to estimate LLRs i.e., TL *vs*. SL; TL *vs.* FL and SL *vs.* FL relationships (Hossain *et al*., 2006a).

2.5. Condition Factors and Form Factor ($a_{3.0}$)

The allometric condition (K_A) was estimated by the equation of Tesch (1968): W/L^b, where *W* is the BW (g), *L* is the TL (cm), and *b* is the LWR parameter. The Fulton's Condition Factor (K_F) was determined by the equation, $K_F =100\times (W/L^3)$, where *W* is the BW (g), and *L* is the TL in cm (Fulton, 1904). The scaling factor of 100 was used to bring the K_F close to unit. Moreover, the relative condition (K_R) was calculated by the equation of Le Cren (1951): $K_R = W/(a\times L^b)$, where *W* is the BW (g), *L* is the TL (cm), and *a* and *b* are LWRs parameters. For assessing W_R, the equation of Froese (2006): $W_R = (W/W_s)\times 100$ was used, where *W* is the weight of a particular individual and W_s is the expected standard weight for the same individual. Additionally, the $a_{3.0}$ of *L. bata* was estimated through the equation of Froese (2006) as: $a_{3.0} = 10^{log\ a\text{-}s(b\text{-}3)}$, where *a* and *b* are regression parameters of LWRs and *s* is the regression slope of *ln a vs. b*. In this study, a mean slope *S* = -1.358, was used for calculating the form factor to estimate of the regression (S) of *ln a vs. b*, since information on the LWRs for this species is unavailable

2.6. Size at Sexual Maturity (L_m) *and Natural Mortality* (M_W)

The L_m of *L. bata* was estimated via the equation, log (L_m) = -0.1189 + 0.9157* log (L_{max}) by Binohlan and Froese (2009) for males and females, separately. Also the M_W was determined using the model, M_W = 1.92 year^{-1} *$(W)^{-0.25}$ (Peterson and Wroblewski, 1984), where, M_W = natural mortality at mass *W*, and $W = a*L^b$, *a* and *b* are regression parameters of LWRs.

2.7. Statistical Analyses

The Microsoft® Excel-add-in-DDXL and GraphPad Prism 6.5 software were used in statistical analysis of this study. The homogeneity and normality of data were checked. The non-parametric Spearman Rank Test was employed to assess the relationship between the morphometric indices (*e.g*., TL, FL, SL, and BW) with condition factors. The Mann-Whitney U-test was employed to compare the mean values between sexes. Furthermore, ANCOVA was utilized for the comparison of LWRs between male and female. The Wilkoxon Sign Ranked Test was applied to evaluate the mean W_R with 100 (Anderson and Neumann, 1996). All statistical analyses were considered significant at 5% (p<0.05).

3. Results

3.1. Sex ratio

This study revealed that, out of the 157 individuals of *L. bata*, sixty-nine (43.95%) were males, and eighty-eight (56.05%) were females. The male and female ratio was 1:1.28; the whole sex ratio did not differ statistically from the expected 1:1 ratio (df=1, χ^2=2.30, p>0.05) (Table 1). However, the deviation in the sex ratio with length class illustrated that both males and females were dominated for 8.00-8.99 cm to 8.99-9.99 cm TL size groups, respectively, though statistically, there was no considerable variations among these groups (p>0.05).

Table 1. Number of male, female and sex ratio (male: female=1:1) of *Labeo bata* (Hamilton, 1822) from the Ganges River, northwestern Bangladesh.

Length class (TL, cm)	Number of specimens			Sex ratio (Male/Female)	χ^2 (*df*=1)	Significance
	Male	Female	Total			
7.00 – 7.99	0	1	1	-	1.00	*ns*
8.00 – 8.99	11	13	24	1:1.18	0.17	*ns*
9.00 – 9.99	12	13	25	1:1.08	0.04	*ns*
10.00 – 10.99	5	6	11	1:1.2	0.09	*ns*
11.00 – 11.99	3	4	7	1:1.33	0.14	*ns*
12.00 – 12.99	3	4	7	1:1.33	0.14	*ns*
13.00 – 13.99	4	3	7	1:0.75	0.14	*ns*
14.00 – 14.99	2	5	7	1:2.5	1.29	*ns*
15.00 – 15.99	2	3	5	1:1.5	0.20	*ns*
16.00 – 16.99	3	5	8	1:1.67	0.50	*ns*
17.00 – 17.99	3	4	7	1:1.33	0.14	*ns*
18.00 – 18.99	3	4	7	1:1.33	0.14	*ns*
19.00 – 19.99	4	4	8	1:1	0.00	*ns*
20.00 – 20.99	3	4	7	1:1.33	0.14	*ns*
21.00 – 21.99	5	4	9	1:0.80	0.11	*ns*
22.00 – 22.99	2	4	6	1:2	0.67	*ns*
23.00 – 23.99	2	4	6	1:2	0.67	*ns*
24.00 – 24.99	2	1	3	1:0.50	0.33	*ns*
25.00 – 25.99	-	2	2	-	2.00	*ns*
Overall	69	88	157	1:1.28	2.30	*ns*

TL, total length; *df*, degree of freedom; *ns*, not significant

3.2. Length-Frequency Distributions (LFDs)

Table 2 illustrates the descriptive statistics for length and weight measurements of the *L. bata.* The LFDs point out that the smallest and largest specimens were 8.00 to 24.3 cm TL for males and 7.9 to 25.2 cm TL for females (Figure 1). The LFDs for both males and females did not pass the normality (Shapiro- Wilk Normality Test, $p<0.001$). In addition, the Mann-Whitney U-test indicates that there was no noteworthy variations in the LFDs between sexes (U= 2872, $p=0.056$). Furthermore, the results showed that, BW of males and females did not pass the normality test (Shapiro- Wilk Normality Test, $p<0.001$), also the Mann-Whitney U-test indicated no significant differences in the BW between sexes (U= 2818, $p>0.05$).

Table 2. Descriptive statistics and estimated parameters of the length-weight relationships ($BW= a \times TL^b$) of *Labeo bata* (Hamilton, 1822) from the Ganges River, northwestern Bangladesh.

Equation	Sex	*n*	Length (cm)		Body weight (g)		Regression parameters		95% CL of *a*	95% CL of *b*	r^2	GT
			Min	Max	Min	Max	*a*	*b*				
$BW=a \times TL^b$	M	69	8.0	24.3	4.68	175.57	0.0070	3.14	0.0062 – 0.0080	3.09 – 3.19	0.996	+A
$BW=a \times FL^b$			7.0	22.1			0.0120	3.08	0.0100 – 0.0144	3.01 – 3.16	0.990	I
$BW=a \times SL^b$			6.2	20.3			0.0215	2.99	0.0178 – 0.0260	2.91 – 3.07	0.988	I
$BW=a \times TL^b$	F	88	7.9	25.2	4.94	181.35	0.0085	3.08	0.0076 – 0.0094	3.04 – 3.12	0.996	+A
$BW=a \times FL^b$			6.9	22.2			0.0153	3.00	0.0128 – 0.0182	2.93 – 3.07	0.988	I
$BW=a \times SL^b$			6.0	19.8			0.0243	2.95	0.0200 – 0.0295	2.87 – 3.03	0.984	-A
$BW=a \times TL^b$	C	157	7.9	25.2	4.68	181.35	0.0078	3.11	0.0072 – 0.0084	3.07 – 3.14	0.996	+A
$BW=a \times FL^b$			6.9	22.2			0.0137	3.04	0.0120 – 0.0155	2.99 –3.09	0.989	I
$BW=a \times SL^b$			6.0	20.3			0.0229	2.97	0.0200 – 0.0362	2.91 – 3.03	0.986	-A

TL, total length; FL, fork length; SL, standard length; BW, body weight; M, male; F, female; C, combined; *n*, sample size; Min, minimum; Max, maximum; *a* and *b* are regression parameter; CL, confidence limit for mean values; r^2, coefficient of determination; GT, growth type; +A, positive allometric; -A, negative allometric; I, isometric

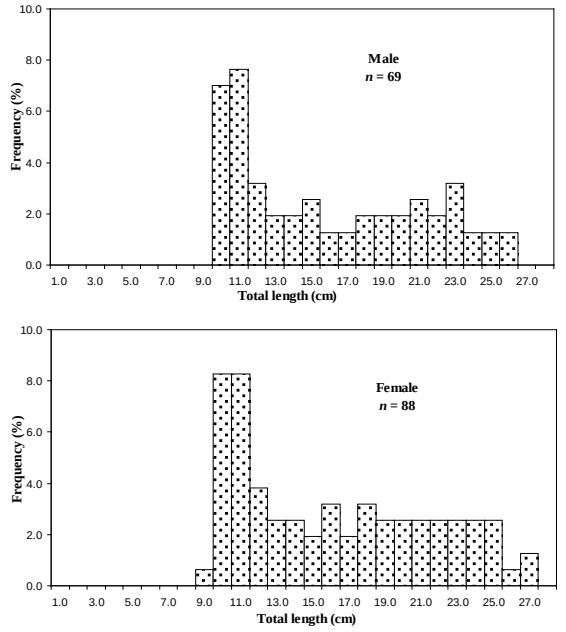


Figure 1. Length-frequency distribution of male and female *Labeo bata* from the Ganges River, northwestern Bangladesh

3.3. Growth Pattern

The sample sizes (*n*), regression parameters and 95% confidence limits for *a* and *b* of the LWRs, coefficients of determination (r^2), and growth type of *L. bata* are shown in Figure 2 and in Table 2. All LWRs were highly significant (p<0.01), with all r^2 values ≥ 0.984. The ANCOVA expressed no considerable differences in LWRs between sexes (p>0.05). Moreover, the LLRs i.e., TL *vs*. SL, TL *vs*. FL and SL *vs*. FL of the *L. bata* along with the estimated parameters, and the coefficient of determination (r^2) are presented in Table 3. All LLRs were highly significant (p<0.001) and most of the r^2 values exceeded 0.992.

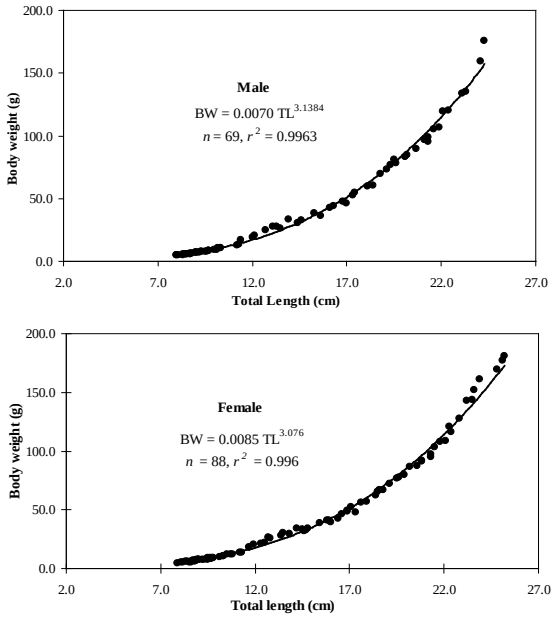


Figure 2. The length-weight relationships (BW= a * TL^b) of male and female *Labeo bata* from the Ganges River, northwestern Bangladesh

Table 3. The estimated parameters of the length-length relationships ($Y = a + b \times X$) of *Labeo bata* (Hamilton, 1822) from the Ganges River, northwestern Bangladesh.

Equation	Sex	Regression parameters		95% CL	95% CL	r^2
		a	*b*	of *a*	of *b*	
TL = *a* + *b* × SL	M	0.8775	1.18	0.5898-1.1651	1.16-1.21	0.993
TL = *a* + *b* × FL		0.4018	1.10	0.1703-0.6334	1.08-1.12	0.996
SL = *a* + *b* × FL		-0.3750	0.93	-0.5053 to -0.2447	0.91-0.94	0.998
TL = *a* + *b* × SL	F	0.6887	1.213	0.3949-0.9825	1.18-1.24	0.991
TL = *a* + *b* × FL		0.5121	1.10	0.2857-0.7386	1.08-1.11	0.995
SL = *a* + *b* × FL		-0.1052	0.90	-0.2610 to 0.0506	0.89-0.91	0.997
TL = *a* + *b* × SL	C	0.7591	1.20	0.5717-0.9466	1.19-1.22	0.994
TL = *a* + *b* × FL		0.4881	1.10	0.3310-0.6452	1.08-1.11	0.995
SL = *a* + *b* × FL		-0.1957	0.91	-0.3038 to -0.0875	0.90-0.92	0.997

TL, total length; FL, fork length; SL, standard length; M, male; F, female; C, combined; *a*, intercept; *b*, slope; CL, confidence limit of mean values; r^2, coefficient of determination

3.4. Condition Factors and Form Factor ($a_{3.0}$)

The different condition factors (K_A, K_F, K_R and W_R) for both sexes of the *L. bata* are given in Table 4. The relationship between TL *vs.* W_R is shown in Figure 3, and the relationships of different condition factors (K_A, K_F, K_R and W_R) with TL and BW are shown in Table 5. Additionally, the calculated $a_{3.0}$ of both sexes of the *L. bata* was 0.0108 (Table 6)·

Table 4. Allometric (K_A), Fulton's (K_F), relative condition factors (K_R) and relative weight (W_R) of *Labeo bata* (Hamilton, 1822) from the Ganges River, northwestern Bangladesh.

Condition factors	Sex	*n*	Min	Max	Mean ± SD	95% CL
K_A	M	69	0.0064	0.0087	0.0071 ± 0.0005	0.0069 – 0.0072
K_F			0.8797	1.2440	1.0114 ± 0.0904	0.9897 – 1.0331
K_R			0.9165	1.2359	1.0097 ± 0.0752	0.9916 – 1.0277
W_R			91.651	123.594	100.968 ± 7.517	99.162 – 102.774
K_A	F	88	0.0070	0.0110	0.0085 ± 0.0006	0.0083 – 0.0086
K_F			0.8930	1.2859	1.0350 ± 0.0787	1.0184 – 1.0517
K_R			0.8797	1.2471	0.9973 ± 0.0709	0.9823 – 1.0123
W_R			87.966	124.707	99.732 ± 7.090	98.230 – 101.234
K_A	C	157	0.0070	0.0100	0.0080 ± 0.0010	0.0077 – 0.0079
K_F			0.8800	1.2900	1.0200 ± 0.0800	1.0100 – 1.0400
K_R			0.9800	1.4100	1.1100 ± 0.0800	1.1000 – 1.1300
W_R			98.340	140.670	111.250 ± 8.220	109.950 –12.540

n, sample size; Min, minimum; Max, maximum; SD, standard deviation; CL, confidence limit for mean values; K_A, allometric condition factor; K_F, Fulton's condition factor; K_R, relative condition factor; W_R, relative weight

Table 5. Relationships of condition factors with total length (TL) and body weight (BW) of *Labeo bata* from the Ganges River, northwestern Bangladesh.

Condition factor	Sex	r_s value	95% CL of r_s	p value	Degree of significance
TL *vs.* K_A	M	0.0559	-0.1902 to 0.2953	$p = 0.648$	*ns*
TL *vs.* K_F		0.6826	0.5266 to 0.7941	$p < 0.001$	***
TL *vs.* K_R		0.0555	-0.1905 to 0.2950	$p = 0.650$	*ns*
TL *vs.* W_R		0.0579	-0.1883 to 0.2971	$p = 0.637$	*ns*
BW *vs.* K_A		0.0746	-0.1720 to 0.3124	$p = 0.542$	*ns*
BW *vs.* K_F		0.6964	0.5454 to 0.8036	$p < 0.001$	***
BW *vs.* K_R		0.0750	-0.1716 to 0.3126	$p = 0.540$	*ns*
BW *vs.* W_R		0.0767	-0.1699 to 0.3143	$p = 0.531$	*ns*
TL *vs.* K_A	F	0.0069	-0.2221 to 0.2089	$p = 0.949$	*ns*
TL *vs.* K_F		0.4843	0.3002 to 0.6337	$p < 0.001$	***
TL *vs.* K_R		-0.0055	-0.2207 to 0.2202	$p = 0.960$	*ns*
TL *vs.* W_R		-0.0041	-0.2194 to 0.2116	$p = 0.970$	*ns*
BW *vs.* K_A		0.0134	-0.2027 to 0.2282	$p = 0.901$	*ns*
BW *vs.* K_F		0.5019	0.3211 to 0.6473	$p < 0.001$	***
BW *vs.* K_R		0.0150	-0.2011 to 0.2298	$p = 0.889$	*ns*
BW *vs.* W_R		0.0166	-0.1996 to 0.2313	$p = 0.878$	*ns*
TL *vs.* K_A	C	0.0586	-0.1036 to 0.2178	$p = 0.466$	*ns*
TL *vs.* K_F		0.5877	0.4711 to 0.6841	$p < 0.001$	***
TL *vs.* K_R		0.0419	-0.1201 to 0.2018	$p = 0.602$	*ns*
TL *vs.* W_R		0.0452	-0.1169 to 0.2050	$p = 0.574$	*ns*
BW *vs.* K_A		0.0871	-0.0751 to 0.2449	$p = 0.279$	*ns*
BW *vs.* K_F		0.6084	0.4958 to 0.7009	$p < 0.001$	***
BW *vs.* K_R		0.0705	-0.0917 to 0.2292	$p = 0.380$	*ns*
BW *vs.* W_R		0.0740	-0.0883 to 0.2325	$p = 0.357$	*ns*

r_S, coefficient of spearman rank correlation test; *ns*, not significant; ***, highly significant

Table 6. The calculated form factor ($a_{3.0}$), size at first sexual maturity (L_m) of *Labeo bata* from the Ganges River, northwestern Bangladesh.

Sex	n	Length (cm)			a	b	$a_{3.0}$	L_m	95% CL of L_m
		Type	Min	Max					
Male	69	TL	8.0	24.3	0.0070	3.14	0.0108	14.12	11.07 - 17.98
Female	88	TL	7.9	25.2	0.0085	3.08	0.0108	14.60	11.43 - 18.61

n, sample size; TL, total length; Min, minimum; Max, maximum; *a*, intercept; *b*, slope; $a_{3.0}$, form factor; L_m, size at first sexual maturity

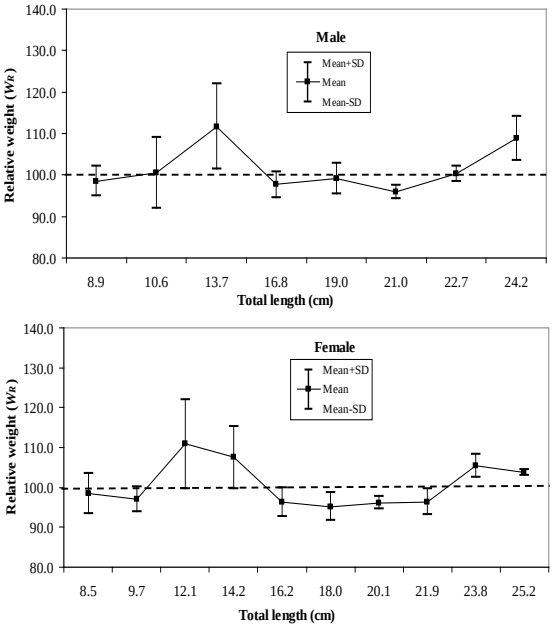


Figure 3. The relationship between total length and relative weight of *Labeo bata* from the Ganges River, northwestern Bangladesh

3.5. Size at First Sexual Maturity (L_m) and Natural Mortality (M_W)

The calculated L_m for male and female populations of *L. bata* was 14.12 cm and 14.60 cm in TL, respectively (Table 6). The M_w for the population of *L. bata* was estimated 0.86 year^{-1} in the Ganges River in northwestern Bangladesh (Figure 4).

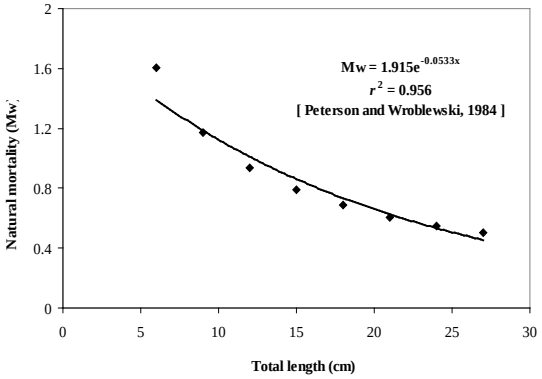


Figure 4. The relationships between total length and natural mortality of *Labeo bata* from the Ganges River, northwestern Bangladesh

4. Discussion

The population parameters are valuable approaches for the evaluation of the status of fish stocks and are vital means for the management of exploited fish populations (Sparre and Venema, 1997). Information on the population parameters of the *L. bata* is very deficient, therefore, the present study provides a complete description on the above-mentioned issue including- growth patterns, condition factors, form factor, reproduction and mortality using a number of individual *L. bata* collected from the Ganges River in NW Bangladesh.

In the current study, the females were more dominant than the males with the sex ratio of male and female of the *L. bata* being 1:1.28, which is statistically not different from the anticipated value of 1:1 (χ^2=2.30, *df*=1, *p*>0.05). Fumio (1960) stated that an increase in the sex ratio in relation to body size for some fish species may be attributed to the high mortality rate in males, and the greater longevity of females.

During the study, *L. bata* with < 7.90 cm TL could not be collected as a result of possible conditions, including the outcomes of the selectivity of the fishing gears, the inability of the fishermen to reach where the smaller size were (Hossain *et al*., 2012a; 2016b), the indiscriminate killing of fry and fingerlings (Rema Devi and Ali, 2013), or because of the spawning season (Rahman, 2005). In the present study, the maximum length found of the *L. bata* was 25.2 cm TL, which is lower than the maximum documented value of 61.0 cm TL (Talwar and Jhingran, 1991). Maximum length is important for estimating the asymptotic length and the growth coefficient of fishes (Azad *et al*., 2018; Hossain *et al*., 2012b; Hossen *et al*., 2016; Nawer *et al*., 2017).

The accepted range of the *b* value is 2.5-3.5 (Froese, 2006). In the present study, all the *b* values for different LWRs were within this limit (2.95-3.14). According to Tesch (1971), when the *b* value is close to 3, it signifies that the fish grow isometrically. The 3.0 signifies an allometric growth, (>3 means positive allometric, and <3 means negative allometric). In this study, the *b* values for TL *vs*. BW relationships were >3.00 (3.08-3.14) indicating a positive allometric growth for males, females and the combined sexes of *L. bata* in the Ganges River, NW Bangladesh. Hossain *et al*. (2016a) reported positive allometric growth for the *L. bata* of the Ganges River, which is similar with the present findings regarding the TL *vs.* BW relationships. Naeem *et al*. (2012) reported negative allometric growth (*b* = 2.92 for combined sexes) for the same species in the Head Panjnad in Pakistan (see Fig. 5), which were inconsistent with the findings in this study. However, the differences in the *b* values may be attributed to the habitat condition, seasonal effects, degree of stomach fullness, gonad maturity, sex, fish health, preservation techniques, and differences in the observed length (Hossain *et al.*, 2006b, 2013a, b, 2014; Tesch, 1971), which we did take into consideration during the study.

All LLRs were highly significant (p<0.001), and most of the r^2 values were ≥0.992 and were dissimilar with the results of Naeem *et al*. (2012). Such variations arise because of differences in the ecological conditions of the habitats, or as a result of the variation in the physiology of animals (Le Cren, 1951), or because the length ranges and the sampling times were not similar.

In the current study, four condition factors (K_A, K_F, K_R and W_R) were used to know the physical and environmental conditions of the *L. bata* in the Ganges River. The Spearman Rank Correlation Test illustrated that only K_F is strongly correlated with TL and BW for both sexes (Table 5) indicating that the Fulton′s Condition Factor (K_F) is the best biometric index for establishing the welfare of *L. bata* in the Ganges River and the adjacent ecosystems. Because this is the first assessment of the condition factor for the *L. bata,* it was not possible to compare the findings of this study with other findings from the same habitat or elsewhere.

The W_R helps to assess the overall health and fitness, as well as the population-level responses to the ecosystem disturbance (Rypel and Richter, 2008). In the present study, according to the Wilkoxon Sign Ranked Test, the W_R showed no significant differences from 100 for both males and females indicating that the population of the *L. bata* in the Ganges River ecosystem is in an equilibrium state with the availability of food and a lower numbers of predators. However, the lack of available information in the literature dealing with the W_R of the *L. bata* limits the comparison of the findings reported in this study to other research findings.

In this study, the $a_{3.0}$ for both the male and female populations of *L. bata* was 0.0108. The $a_{3.0}$ can be used to verify whether the body profile of individuals in a particular population is considerably different from others (Froese, 2006).

Also, the L_m for male and female *L. bata* populations was 14.12 cm and 14.60 cm in TL, respectively. Studies on L_m for Bangladeshi freshwater fishes are very rare (except Azadi and Naser, 1996; Hossain *et al*., 2010; 2012c; 2016b,c; 2017). The L_m is broadly used as an indicator for minimum allowable capture size (Lucifora *et al*., 1999). This research presents the first attempt to determine the L_m for the *L. bata* of the Ganges River. Hopefully this will be the foundation for more detailed studies to investigate the factors affecting the first sexual maturation and spawning size.

The M_w for the population of the *L. bata* was estimated 0.86 year^{-1} in the Ganges River, NW Bangladesh. Since this is the first assessment of the M_w for this species, it was not possible to compare the findings with other studies. Therefore, further studies are recommended to find out the reasons of mortality of this species in the future.

5. Conclusion

The present work is the first of its kind to provide fundamental information on the wild population of the *L. bata*, including sex ratio and length-frequency distribution, length-weight and length-length relationships, condition factors (allometric, Fulton's, relative and relative weight), form factor, first sexual maturity and natural mortality. The findings of this study would be a baseline for developing and controlling the *L. bata* exploitation, to achieve an effective and sustainable management of this fishery in the Ganges River of NW Bangladesh, as well as in other subtropical countries.

Acknowledgements

The authors would like to extend their sincere appreciation to the (i) NATP-2, PIU-BARC, Sub-project ID: 484 (Ref: NATP-2/PIU-BARC-24/Research/2016/765; Date: 04-07-2017) (ii) Ministry of Education, Bangladesh (No. 37.200000.004.003.005.2014-1309/1 (42); Date: 10-08-2014) and (iii) IDRSBFRI, Bangladesh Fisheries Research Institute, Shrimp Research Institute, Bagerhat, Bangladesh (Serial No. 04, Session: 2013-2014) for providing funding to carry out this research.

Conflicts of interest

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

References

Anderson RO and Neumann RM. 1996. Length, weight and associated structure indices**. In: Fisheries Techniques**, 2nd ed., Murphy B.R. and W. Willis, D. (Eds). American Fisheries Society, Bethesda, Maryland, pp.447-482.

Azad MAK, Hossain MY, Khatun D, Parvin MF, Nawer F, Rahman O and Hossen MA. 2018. Morphometric Relationships of the Tank goby *Glossogobius giuris* (Hamilton, 1822) in the Gorai River using Multi-linear Dimensions. *Jordan J Biol Sci*, **11**: 81-85.

Azadi MA and Naser A. 1996. Length-weight relationship and relative condition factor of a carp, *Labeo bata* (Ham.) from Kaptai reservoir, Bangladesh. *Chittagong Univ Stud*, P-II, **20**:19-25.

Binohlan C and Froese R. 2009. Empirical equations for estimating maximum length from length at first maturity. *J Appl Ichthyol*, **25**: 611-613.

Chen Y and Paloheimo JE. 1994. Estimating fish length and age at 50% maturity using a logistic type model. *Aquat Sci*, **56**: 206-219.

Froese R and Pauly D. (Eds). 2016. **Fish Base 2016**. World Wide Web electronic publication. Available at: http://www.fishbase.org (accessed on 10 July 2016).

Froese R. 2006: Cube law, condition factor and weight-length relationships: History, meta-analysis and recommendations. *J Appl Ichthyol*, **22**: 241-253.

Fulton TW. 1904. **The Rate of Growth of Fishes**. *Twenty-second Annual Reports*, Part III. Fisheries Board of Scotland, Edinburgh, pp.141-241.

Fumio M. 1960. Fishery biology of the yellow-tail, *Serioa quinqueradiata*, inhabiting the waters surrounding Japan. *Mem Facul Agric Kinki Univ*, **1**: 300.

Hossain MY, Ahmed ZF, Islam, ABMS, Jasmine S and Ohtomi J. 2010. Gonadosomatic index based size at first sexual maturity and fecundity indices of the Indian River shad *Gudusia chapra* (Clupeidae) in the Ganges River (NW Bangladesh). *J Appl Ichthyol*, **26:** 550-553.

Hossain MY, Ahmed ZF, Leunda PM, Islam AKMR, Jasmine S, Oscoz J, Miranda R and Ohtomi J. 2006a. Length-weight and length-length relationships of some small indigenous fish species from the Mathabhanga River, southwestern Bangladesh. *J Appl Ichthyol*, **22**: 301-303.

Hossain MY, Ahmed ZF, Leunda PM, Jasmine S, Oscoz J, Miranda R and Ohtomi J. 2006b. Condition, length weight and length-length relationships of the Asian striped catfish *Mystus vittatus* (Bloch, 1794) (Siluriformes: Bagridae) in the Mathabhanga River, southwestern Bangladesh. *J Appl Ichthyol*, **22:** 304-307.

Hossain MY, Ohtomi J, Ahmed J, Jasmine S and Vadas RL. 2012a. Life-history traits of the Monsoon River prawn 1844) (Palaemonidae) in the Ganges (Padma) River, *Macrobrachium malcolmsonii* (Milne-Edwards, northwestern Bangladesh. *J Freshwat Ecol*, **27:** 131-142.

Hossain MY, Jewel MAS, Nahar L, Rahman MM, Naif A and Ohtomi J. 2012b. Gonadosomatic index-based size at first sexual maturity of the catfish *Eutropiichthys vacha* (Hamilton, 1822) in the Ganges River (NW Bangladesh). *J Appl Ichthyol*, **28:** 601-605.

Hossain MY, Rahman MM, Miranda R, Leunda PM, Oscoz J, Jewel MAS, Naif A and Ohtomi J. 2012c. Size at first sexual maturity, fecundity, length– weight and length–length relationships of *Puntius sophore* (Cyprinidae) in Bangladeshi waters. *J Appl Ichthyol*, **28:** 818-822.

Hossain MY, Jewel MAS, Rahman MM, Haque ABMM, Elbaghdady HM and Ohtomi J. 2013a. Life-history Traits of the Freshwater Garfish *Xenentodon cancila* (Hamilton 1822) (Belonidae) in the Ganges River, Northwestern Bangladesh. *Sains Malays*, **42**: 1207–1218.

Hossain MY, Rahman MM, Jewel MAS, Hossain MA, Ahamed F, Tumpa AS, Abdallah EM and Ohtomi J. 2013b. Life-history traits of the critically endangered catfish *Eutropiichthys vacha* (Hamilton, 1822) in the Jamuna (Brahmaputra River distributary) River, northern Bangladesh. *Sains Malays*, **42:** 265-277.

Hossain MY, Rahman MM, Ahamed F, Ahmed ZF and Ohtomi J. 2014. Length-weight and length-length relationships and form factor of three threatened fishes from the Ganges River (NW Bangladesh). *J Appl Ichthyol*, **30:** 221–224.

Hossain MY, Hossen MA, Pramanik MNU, Yahya K, Bahkali AH, Elgorban AM. 2016a. Length-weight relationships of *Dermogenys pusilla* Kuhl and van Hasselt, 1823 (Zenarchopteridae) and *Labeo bata* (Hamilton, 1822) (Cyprinidae) from the Ganges River (NW Bangladesh). *J Appl Ichthyol*, **32**:744-746.

Hossain MY, Naser SMA, Bahkali AH, Yahya K, Hossen MA, Elgorban AM., Islam MM and Rahman MM. 2016b. Life history traits of the flying barb *Esomus danricus* (Cyprinidae) in the Ganges River, Northwestern Bangladesh. *Pakistan J Zool*, **48:** 399-408.

Hossain MY, Hossen, MA, Islam, MM, Pramanik, MNU, Nawer, F, Paul, AK, Hameed HMA, Rahman MM, Kaushik, G and Bardoloi S. 2016c. Biometric indices and size at first sexual maturity of eight alien fish species from Bangladesh. *Egyptian J Aquat Res*, **42:** 331-339.

Hossain MY, Hossen MA, Khatun D, Nawer F, Parvin MF, Rahman O and Hossain MA. 2017. Growth, condition, maturity and mortality of the Gangetic Leaffish *Nandus nandus* (Hamilton, 1822) in the Ganges River (Northwestern Bangladesh). *Jordan J Biol Sci*, **10:** 57-62.

Hossen MA, Hossain MY, Yahya K and Pramanik MNU. 2015. Threatened fishes of the world: *Labeo bata*(Hamilton, 1822) (Cypriniformes: Cyprinidae). *Croatian J Fish*, **73:** 89-91.

Hossen MA, Hossain MY, Pramanik MNU, Nawer F, Khatun D, Parvin MF and Rahman MM. 2016. Morphological characters of *Botia lohachata*. *J Coast Life Med*, **4**: 689-692.

IUCN Bangladesh. 2015. **Red List of Bangladesh Volume 5: Freshwater Fishes.** IUCN, International Union for Conservation of Nature, Bangladesh Country Office, Dhaka, Bangladesh, p.xvi+360.

King MG. 1997. **Fisheries Biology, Assesment and Management**. Fishing news books. Oxford: Osney Mead. p.341.

Le Cren E. D. 1951. The length-weight relationship and seasonal cycle in gonad weight and condition in the perch (*Perca fluviatilis*). *J Animl Ecol*, **20**: 201-219.

Lucifora LO, Valero JL and Garcia VB. 1999. Length at maturity of the green-eye spurdog shark, *Squalus mitsukuii* (Elasmobranchii: Squalidae) from the SW Atlantic, with comparisons with other regions. *Mar Freshwater Res*, **50**: 629-632.

Naeem M, Zuberi A, Khan NA, Rasool SA, Ismail HF and Qamar A. 2012. Some morphometric relationship traits of *Labeo bata* (Hamilton, 1822) from Head Panjnad, Pakistan. *Afr J Biotechnol*, **11**: 15465-15468.

Nawer F, Hossain MY, Hossen MA, Khatun D, Parvin MF, Ohtomi J and Islam MA. 2017. Morphometric Relationships of the Endangered Ticto barb *Pethia ticto* (Hamilton, 1822) in the Ganges River (NW Bangladesh) through Multi-Linear Dimensions. *Jordan J Biol Sci*, **10**: 199–203.

Peterson I and Wroblewski JS. 1984. Mortality rates of fishes in the pelagic ecosystem. *Can J Fish Aquat Sci*, **41**:1117–1120.

Rema Devi KR and Ali A. 2013. *Labeo bata*. The IUCN Red List of Threatened species. Version 2014.1. <www.iucnredlist.org> (Accessed on: 10 October 2017).

Robins CR, Bailey RM, Bond CE, Brooker JR, Lachner EA, Lea RN and Scott WB**.** 1991. **World Fishes Important to North Americans. Exclusive of Species from the Continental Waters of the United States and Canada**. American Fisheries Society Special Publication 21. American Fisheries Society, Bethesda, MD. p.243.

Rahman AKA. 2005. **Freshwater Fishes of Bangladesh,** 2nd edition. Zoological Society of Bangladesh, Department of Zoology, University of Dhaka, Dhaka-1000, pp. 1-263.

Rypel AL and Richter TJ. 2008. Empirical percentile standard weight equation for the black tail red horse. *North Am J Fish Manage*, **28**: 1843-1846.

Siddique AQ, Chatterjee A and Khan AA. 1976. Reproductive biology of the carp, *Labeo bata* (Ham.) from the River Kali, India. *Aquaculture*, **7:**181–191.

Sokal RR and Rohlf FJ. 1987. **Introduction to Biostatistics**. 2nd Ed. New York: Freeman Publication.

Sparre P and Venema SC. 1997. *Introdução à avaliação de mananciais de peixes tropicais*. Roma: FAO. Documento Técnico Sobre as Pescas no. 306/1.

Talwar PK and Jhingran AG. 1991. **Inland Fishes of India and Adjacent Countries**. vol 1. A.A. Balkema, Rotterdam. p.541.

Tesch FW. 1968. Age and growth**. In: Methods for Assessment of Fish Production in Fresh Waters**, Ricker, W.E. (Eds). Oxford: Blackwell Scientific Publications.

Tesch FW. 1971. Age and Growth**. In: Methods for Assessment of Fish Production in Fresh Waters**, Ricker, W.E.(Eds). Oxford: Blackwell Scientific Publications. pp.98-130.

JJBS

Volume 11, Number 2,June 2018
ISSN 1995-6673
Pages 187 - 194

Jordan Journal of Biological Sciences

Submerged Fermentation of Orange Albedo to Produce Gibberellic Acid Using *Fusarium moniliforme* and *Aspergillus niger*

Patricia F. Omojasola* and Damola O. Adejoro

Department of Microbiology, Faculty of Life Sciences, University of Ilorin, P.M.B. 1515, Ilorin, Kwara State, Nigeria.

Received October 7, 2017; Revised November 11, 2017; Accepted November 17, 2017

Abstract

This study investigated the potential of orange albedo, an agro-industrial waste, as a suitable substrate for the production of gibberellic acid (GA) through submerged fermentation using *Fusarium moniliforme* ATCC 10052 and *Aspergillus niger* CBS 513.88 due to the high cost of synthetic and plant-extracted GA. The orange fruits were washed and the albedo removed. The albedo was dried, ground, and its proximate composition was determined. The ground orange albedo was incorporated into a modified CzapekDox medium and was fermented using the test fungi. Carboxymethyl cellulose (CMC) served as control. Fermentation conditions were: pH 5.5; inoculum size, 1 mL (5 x 10^5 CFU/mL *F. moniliforme*) (2 x 10^6 CFU/mL *A. niger*); substrate concentration 2 g; temperature 25±2 $^{\circ}$C for seven days. Fermentation was optimized by supplementation with copper sulphate and variation of fermentation conditions. Results of proximate analysis were: moisture 7.46%; crude protein 4.69%; lipids 0.62%; ash 2.41%; crude fibre 27.67%; and carbohydrate 57.15%. GA yield by *F. moniliforme* and *A. niger* on the orange albedo substrate was 5.53 g/L and 6.33 g/L respectively. This increased to 9.39 g/L by *F. moniliforme* and 7.42 g/L by *A. niger* after optimization. These results support the suitability of orange albedo as a promising cheap substrate production of GA.

Keywords: Orange albedo, gibberellic acid, fermentation, *Aspergillus niger, Fusarium moniliforme*, fruit waste

1. Introduction

Gibberellins are isoprenoid phytohormones which play important roles in early germination processes of plants by activating enzyme production and mobilizing storage reserves (Rademacher, 2016). Gibberellic acid (GA) is one of the most important members of the gibberellins due to its industrial and agricultural applications (Rodrigues *et al.,* 2009). Over 120 members of this group of phytohormones have been identified and structurally characterized using chemical and spectroscopic methods (Macmillan, 2002). Among the gibberellins, the ones that have been reported as bioactive are GA_1, GA_3, GA_4, and GA_7. The biologically-inactive gibberellins occur in plants as precursors for the synthesis of the bioactive ones (Yamaguchi, 2008). Gibberellic acid (gibberellin A_3 or GA_3) is one of the most important members of the bioactive gibberellins due to its industrial and agricultural applications (Ates *et al*., 2006).

Gibberellic and abscisic acids are endogenous growth-regulating hormones which control the breaking of seed dormancy to germination alongside other factors such as light, temperature, moisture, and nutrients (Gupta and Chakrabarty, 2013). While GA stimulates seed germination, abscisic acid on the other hand, is concerned with the establishment and maintenance of dormancy. GA has been used extensively for the promotion of crop yields, resistance to pest, alleviation of plant stress, reduction in fruit spoilage and the reduction in flowering times of ornamental plants (Barani *et al*., 2013; Akter *et al*., 2014; Alrashdi *et al*., 2017; Alvarenga *et al*., 2017). However, its high cost has restricted its application to the growth-promotion of plants with high economic values. While GA can be isolated from some tissues in plants, it is a difficult process often marked by poor yields which may be as low as 38 mg/tonne of plant tissue (Mander, 2003). In a similar manner, the production of gibberellins through chemical synthesis is very complicated and unprofitable for industrial applications (Rademacher, 2016). Research has been geared to finding wider GA applications in agriculture and plant biotechnology (Shukla *et al*., 2005; Da Silva *et al*., 2013). Hence, there is a need to utilize cheap substrates for the production of GA.

Oranges (*Citrus sinensis*) are grown in more than 125 countries, and the worldwide production for 2016/17 was estimated at 50.2 million tonnes (USDA, 2017). Apart from fruit-processing industries, oranges are also consumed for their fleshy fruit and juice, after which the peel and albedo are discarded into the environment. Citrus

* Corresponding author. e-mail: folakejasola@yahoo.co.uk; jasola@unilorin.edu.ng
** Abbreviations: CMC (Carboxymethyl cellulose), GA (Gibberellic acid), OA (Orange albedo).

peel waste comprises about 50% of the fresh weight of the fruit (Rodriguez-Fernandez *et al*., 2011). The orange -juice processing industries produce significant volumes of wastes made up of soluble and insoluble carbohydrates (Zhou *et al*., 2011). The disposal of fruit wastes poses considerable environmental and economic problems (Bezalwar *et al*., 2013). However, the utilization of these wastes for fermentation purposes will not only reduce their potentially deleterious effects on the environment, but will also serve as cheap carbon sources for the industrial production of value-added products (Rivas *et al.,* 2008, Torrado *et al.,* 2011; Omojasola and Benu, 2016).

Industrially, GA is produced largely by a submerged fermentation technique using *Gibberella fujikuroi* (renamed *Fusarium fujikuroi*), the perfect stage of *Fusarium moniliforme* (Bruckner and Blechschmidt, 1991; O'Donnell *et al*., 1998; Santos *et al*., 2003). Other methods of GA production, which include the chemical synthesis and extraction from plants, are not economically feasible (Sleem, 2013). While solid-state fermentation has been reported to have a potential for higher yields, lower energy consumption, reduced risk of bacterial contamination, lower catabolic repression, and lesser environmental impacts (Viniegra-Gonalez *et al*., 2003; Torrado *et* al., 2011; Rangaswamy, 2012; Zhang *et al*., 2015), however, it is difficult to monitor the fermentation parameters such as pH, inoculum concentration, nutrient composition, dissolved oxygen composition and fermentation time, and to optimize them using solid-state fermentation (Kumar *et al.*, 2011). In addition, submerged fermentation allows an easier purification of the product (Subramaniyam and Vimala, 2012). Some other microorganisms that have been found to produce GA include: *Aspergillus niger*, *Azospirillum, Azotobacter, Bacillus* spp. and *Pseudomonas* spp. (Rademacher, 1994; Cihangir, 2002; Ates *et al*., 2006; Karacoç and Aksöz, 2006; Ambawade and Pathade, 2015). A variety of agro and fruit wastes have been utilized in the production of organic acids using submerged and solid-state fermentation such as pineapple peel, sugarcane baggasse, banana peel to produce citric acid (Kareem and Rahman, 2013; Omojasola *et al.,* 2014), cashew apple juice and corn cob to produce oxalic acid (Betiku *et al.,* 2016; Mai *et al.,* 2016), *Jatropha* seedcake, sweet potato peel to produce itaconic acid (El Imam *et al*., 2013; Omojasola and Adeniran, 2014) Shea nut shell, citric pulp, soy bran, soy husk, cassava bagasse and coffee husk to produce GA (Rodrigues *et al.,* 2009; Kobomoje *et al.,* 2013).

To our knowledge, there is a dearth of data on the suitability of orange peel wastes for the production of GA. Hence, the primary aim of the current work was to study the suitability of orange albedo as a substrate for the production of GA by *F. moniliforme* and *A. niger*.

2. Materials and Methods

2.1. Collection of Samples and Test Organisms

The oranges (*Citrus sinensis*) were procured from the Ipata Market in Ilorin in Kwara State, Nigeria (with coordinates 8.99897 N, 4.561369 E) in November of 2016. The orange fruits were authenticated at the Herbarium Unit of the Department of Plant Biology at the University of Ilorin, with voucher specimen number UILH/001/996. The microorganisms used for the fermentation were *Fusarium moniliforme* ATCC 10052 and *Aspergillus niger* CBS 513.88 obtained from the Microbial Culture Collection of the Department of Microbiology at the University of Ilorin in Nigeria. They were maintained on PDA slants at 4°C to be used later.

2.2. Substrate Preparation

The orange fruits were washed with clean water to remove dirt; after which they were peeled, taking care while separating the peel from the albedo. The albedo was then air-dried for seven days. It was thereafter ground into fine particles (1 mm particle size) by an electric blender (Binatone BLG 699). Then it was stored in a cool and dry place to avoid moisture uptake (Nandini *et al*., 2014).

2.3. Proximate Analysis

The proximate analysis of the substrate was carried out using standard procedures. The parameters investigated were moisture content (Bradley, 2010), lipid, crude fibre, ash, crude protein and carbohydrate contents (AOAC, 1990, 2002).

2.4. Spore Suspension

Fungal spore inoculum was produced by washing spores of a fully-sporulated (7-day old) Potato Dextrose Agar (Difco) slant of each test fungus with 10 mL of sterile distilled water in sterile 250 ml Erlenmeyer flasks. The flasks were then agitated at 150 rpm for thirty minutes for uniform dispersal of spores (Omojasola and Benu, 2016) and adjusted approximately to 5.0×10^5 CFU/mL and 2.0×10^6 CFU/mL for *F. moniliforme* and *A. niger* respectively. The size of the inoculum was determined by counting using the improved Neubauer haemocytometer.

2.5. Fermentation Media

The fermentation medium was a modified CzapekDox broth using the method of Rangaswamy (2012) with replacement of sucrose with orange albedo substrate. The fermentation medium was compounded by adding 2 g of substrate to 100 mL of mineral salts medium. The composition of the mineral salts in 1 litre of water was $NaNO_3$ (3g), K_2HPO_4 (1g), $MgSO_4.7H_2O$ (0.5g), KCl (0.5g), and $FeSO_4$ (0.01g). The fermentation medium was sterilized by autoclaving at 121 °C before use.

2.6. Submerged Fermentation

The test organisms were drawn separately from the spore suspension, and each was inoculated into 100 mL of sterile fermenting medium. The fermentation was carried out at 25±2 °C on a rotary shaker (LH Fermentation, Model Mk V orbital shaker) at 150 rpm for seven days. The final pH was adjusted using 2M NaOH or 1M HCl. The GA production was monitored every twenty-four hours.

2.6.1. Optimization of GA Production

The optimization experiments were conducted varying the following parameters: fermentation period (fermentation was allowed to continue till GA yield began to drop); pH (4.5 - 5.5); inoculum size (1.0 – 2.0%); substrate concentration (1.0 – 3.0g).

2.6.2. Media Supplementation

The effect of copper sulphate ($CuSO_4$) supplementation on GA production was evaluated. Three concentrations of $CuSO_4$ (0.02% w/v, 0.05% w/v, and 0.08% w/v) were added to different fermentation media (Chinedu *et al*., 2011), and the fermentation proceeded under the same conditions as the non-supplemented cultures.

2.6.3. Assay of GA

This was estimated in the supernatant of fermentation media by spectrophotometrically (Searchtech 752N UV-VIS) using a modified method described by Berrios *et al.* (2004) at 254 nm. The amount of gibberellic acid was calculated from the standard curve obtained by dissolving 0.4 g in absolute alcohol, and diluted to 100 ml in a volumetric flask with absolute alcohol. Each series of data obtained from the spectrophotometric measurement was fitted by linear regression analysis using GraphPad Prism software. The calibration graph obtained was used for the determination of the concentration of gibberellic acid with interpolated values after entering the obtained figures of absorbance.

2.6.4. Recovery of GA

GA was recovered from the fermentation media using methods described by Rachev *et al*. (1993), and Ates *et al*. (2006). The fermentation broth was filtered to separate the mycelia from the media. The filtrate was then adjusted to pH 2 - 2.5 with 2 N HCl, and extracted with ethyl acetate (ratio 1:3, filtrate to solvent). The ethyl acetate phase was treated with activated charcoal 1:1.33% (w/v), and re-filtered to remove the activated charcoal. The ethyl acetate phase was extracted with equal volume of saturated $NaHCO_3$ to separate the GA from other organic impurities. This was further acidified to pH 2.5 with 2 N HCl; re-extracted, dried over anhydrous Na_2SO_4 and concentrated to about 2% of its initial volume using a rotary evaporator. The concentrate was kept at 8^oC for crystallisation.

2.7. Data Analysis

Statistical significance was determined using the one-way analysis of variance (ANOVA) and two-way ANOVA, while multiple comparisons between means were determined by Tukey's or Sidak's multiple comparisons test. Analysis was performed using GraphPad Prism software (GraphPad Software Inc. La Jolla, CA, USA), and SigmaPlot for Windows (version 10.0) (SysStatSoftwares Inc.). All data are expressed as means of triplicates ± SEM or SD, and values of (p<0.05) were considered significant, and 'n' represented independent experiments.

3. Results

3.1. Proximate Analysis

The proximate analysis of the orange albedo substrate showed that it contained 7.46% moisture, 4.69% crude protein, 0.62% lipid, 2.41% ash, 27.67% crude fibre and 57.15% carbohydrate (Table 1).

Table 1. Proximate composition of orange albedo.

Moisture Content (%)	Crude Protein (%)	Lipid Content (%)	Ash Content (%)	Crude Fibre (%)	Carbohydrate (%)
7.46±0.02	4.69± 0.18	0.62±0.01	2.41±0.14	27.67±0.45	57.15±0.98

Values represented are means of triplicates ±SEM

3.2. Pre-optimization of GA Production

The GA production by *F. moniliforme* peaked at 5.53 ±0.02 g/L on Day 6, while the maximum yield by *A. niger* 6.30 ±0.01 g/L was on day five (Table 2). The CMC control produced significantly lower (p<0.05) yield than the OA substrate. *F. moniliforme* and *A. niger* produced 3.62 ±0.01 g/L and 2.61 ±0.07 g/L of GA respectively when CMC was used as substrate.

Table 2. Production of gibberellic acid by submerged fermentation of orange albedo using *Fusarium moniliforme* and *Aspergillus niger*..

Time (Days)	Gibberellic acid (g/L)			
	Fusarium moniliforme		*Aspergillus niger*	
	Orange albedo	CMC (Control)	Orange albedo	CMC (Control)
1	0.48±0.05^a	0.14±0.01^b	2.42±0.01^a	0.03±0.01^b
2	0.90±0.12^b	0.91±0.02^a	2.80±0.01^a	0.15±0.01^b
3	2.50±0.61^a	1.73±0.01^a	4.15±0.02^a	0.82±0.02^b
4	1.67±0.80^b	3.62±0.01^a	4.10±0.16^a	1.24±0.11^b
5	2.22±0.01^a	1.88±0.04^b	6.30±0.01^a	2.61±0.07^b
6	5.53±0.02^a	1.78±0.02^b	3.73±0.01^a	2.54±0.01^b
7	5.25±0.13^a	2.63±0.03^b	4.07±0.17^a	1.46±0.03^b

Values represented are means of triplicates ±SEM of amount of gibberellic acid. Means with the same superscript in a column are not statistically different from each other (*F. moniliforme* and *A. niger* were compared separately)

3.3. Optimization of GA Production

To optimize the GA yield, fermentation parameters such as time, pH, inoculum size and substrate concentration were varied.

3.3.1. Effect of Varying Fermentation Time

The GA yield by *F. moniliforme* peaked on the day six of fermentation (5.5 ±0.03 g/L); however, there was no significant difference (p<0.05) in the yields on day six and seven. The highest GA yield of 6.3 ±0.09 g/L by *A. niger* was recorded on day five (Figure 1). Generally, the yields from the OA substrate were higher than the CMC control.

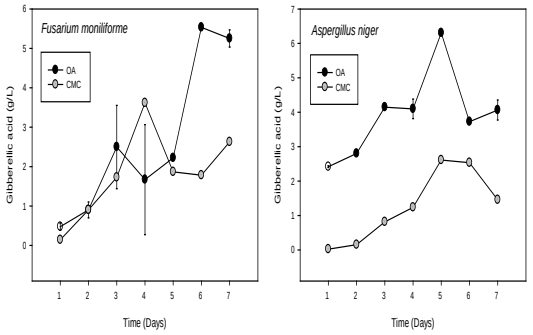


Figure 1. Effect of varying fermentation time on gibberellic acid production by *F. moniliforme* and *A. niger* using orange albedo.

3.3.2. Effect of Varying pH

F. moniliforme produced the highest GA yields at pH 5.0 (12.96 ±0.03 g/L) significantly higher than pre-optimization yields (Figure 1). The lowest peak yield of 5.53 ±0.03 g/L was at pH 5.5. For *A. niger,* pH 5.5 recorded the highest yield of 6.30 ±0.98g/L, while the lowest peak yield of 2.62 ±0.02 g/L was recorded at pH 4.5 (Figure 2).

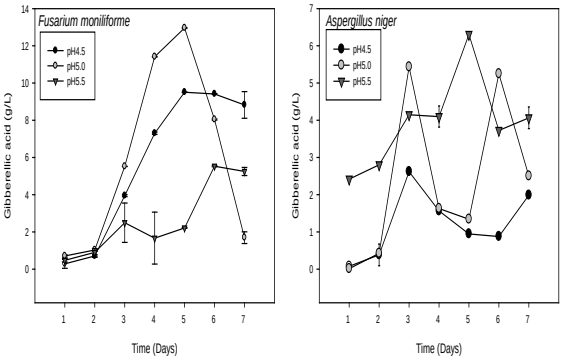


Figure 2. Effect of varying pH on gibberellic acid yield using *F. moniliforme* and *A. niger* grown on orange albedo

3.3.3. Effect of Varying Inoculum Size

The highest GA yield was recorded using 2% inoculum for both fermenting organisms. The yield of *F. moniliforme* was 15.96 ±0.04 g/L and *A. niger* 10.74 ±0.04 g/L (Figure 3). The yield by *F. moniliforme* was the highest yield in this study and was significantly higher ($p<0.05$) than pre-optimized yields.

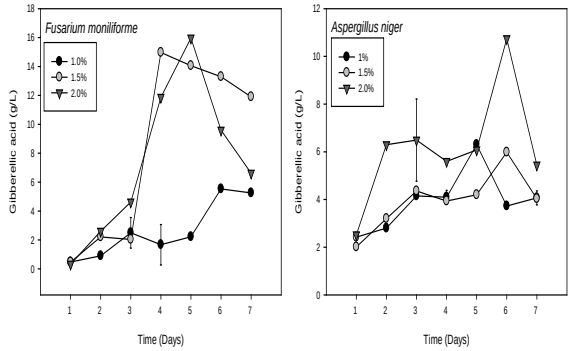


Figure 3. Effect of varying inoculum size on gibberellic acid yield using *F. moniliforme* and *A. niger* grown on orange albedo.

3.3.4. Effect of Varying Substrate Concentration

Peak yields of GA were obtained at substrate concentration of 3 g for both *F. moniliforme* and *A. niger*. *F. moniliforme* yielded 9.47 ±0.09g/L, while *A. niger* yielded 10.78 ±0.08 g/L (Figure 4). These yields were also significantly higher than pre-optimized yields.

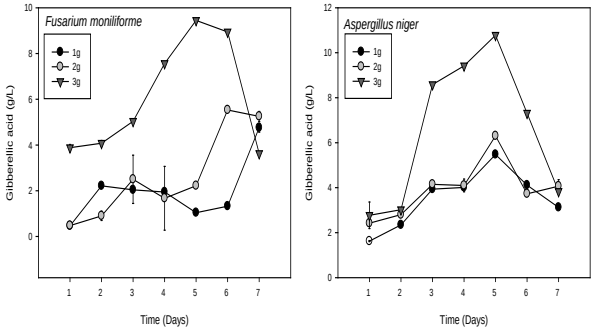


Figure 4. Effect of varying substrate concentration on gibberellic acid yield using *F. moniliforme* and *A. niger* grown on orange albedo.

3.3.5. *Effect of Medium Supplementation*

The yields of gibberellic acid obtained after supplementation with different concentrations of $CuSO_4$ recorded highest GA yields of 5.86 g/L on day seven by *F. moniliforme* using 0.08% $CuSO_4$ and 6.33 g/L on day five by *A. niger* also with 0.08% $CuSO_4$ supplementation (Table 3). It was also observed that there were no significant differences in the yields obtained at 0.02% and 0.08% on day seven for *F. moniliforme* and all the concentrations used on day five for *A. niger* (Table 3).

Table 3. Gibberellic acid production by *F. moniliforme* and *A. niger* grown on orange albedo supplemented with different concentrations of copper sulphate.

Time	Gibberellic acid (g/L)							
(Days)	*Fusarium moniliforme*				*Aspergillus niger*			
	0.02% $CuSO_4$	0.05% $CuSO_4$	0.08% $CuSO_4$	Control	0.02% $CuSO_4$	0.05% $CuSO_4$	0.08% $CuSO_4$	Control
1	0.74± 0.11^a	0.70± 0.03^a	0.70± 0.05^a	0.48± 0.05^a	1.75± 0.23^b	1.42± 0.09^c	2.29± 0.08^a	2.42± 0.01 ^{a}
2	1.34± 0.05^a	1.44± 0.06^a	1.48± 0.04^a	0.90± 0.12^a	3.20± 0.10^a	3.28± 0.18^a	2.80± 0.09^b	2.80± 0.01^b
3	1.52± 0.07^b	1.53± 0.08^b	1.45± 0.04^b	2.50± 0.61^a	4.06± 0.09^a	2.83± 0.07^b	4.15± 0.21^a	4.15± 0.02^a
4	1.99± 0.10^a	1.68± 0.09^a	1.74± 0.11^a	1.67± 0.80^a	4.33± 0.25^b	3.65± 0.05^c	5.29± 0.05^a	4.10± 0.16^b
5	3.21± 0.06^a	3.37± 0.06^a	3.79± 0.04^a	2.22± 0.01^b	6.29± 0.12^a	5.99± 0.20^a	6.33± 0.10^a	6.30± 0.01^a
6	5.53± 0.05^a	4.35± 0.16^b	4.04± 0.10^b	5.53± 0.02^a	5.39± 0.09^a	5.56± 0.17^a	3.70± 0.12^b	3.73± 0.01^b
7	5.55± 0.07^a	4.25± 0.12^b	5.86± 0.05^a	5.25± 0.13^a	3.56± 0.08^b	4.22± 0.23^a	3.47± 0.20^b	4.07± 0.17^b

Values represented are means ±SD of amount of Gibberellic acid. Means with the same superscript across a column are not statistically different from each other (*F. moniliforme* and *A. niger* were compared separately)

3.3.6. Optimized Production of GA

The GA yield under optimized conditions recorded 9.39 ±0.16 g/L by *F. moniliforme* and 7.42 ±0.02 g/L by *A. niger* both on day six of fermentation (Table 4). These were significantly higher (p<0.05) than the peak yields obtained from the CMC control under the same optimized conditions. These yields were higher than those from the pre-optimized fermentations which were 5.53±0.02 g/L and 6.30±0.01 g/L by *F. moniliforme* and *A. niger* respectively (Table 2). In addition, *F. moniliforme* produced higher amounts of GA, although not significant (p<0.05) than *A. niger* under optimized conditions.

Table 4. Production of gibberellic acid by submerged fermentation of orange albedo using *Fusarium moniliforme* and *Aspergillus niger* under optimized conditions.

Time (Days)	Gibberellic acid (g/L)			
	Fusarium moniliforme		*Aspergillus niger*	
	Orange albedo	CMC (Control)	Orange albedo	CMC (Control)
1	1.55±0.03^a	0.91±0.03^b	1.72±0.02^a	1.31±0.03^b
2	4.19±0.02^a	2.75±0.03^b	3.81±0.01^a	1.81±0.03^b
3	5.26±0.03^a	5.25±0.02^a	4.35±0.06^a	2.21±0.03^b
4	5.64±0.03^a	5.56±0.06^a	3.84±0.03^a	2.39±0.03^b
5	5.48±0.01^b	6.84±0.06^a	5.60±0.02^a	3.45±0.04^b
6	9.39±0.16^a	7.03±0.04^b	7.42±0.02^a	4.28±0.04^b
7	8.95±0.05^a	3.80±0.09^b	3.80±0.01^a	1.85±0.03^b

Values represented are means ±SEM of gibberellic acid produced. Means with the same superscript in a row are not statistically different from each other (*F. moniliforme* and *A. niger* were compared separately)

4. Discussion

Fruit wastes constitute part of the most abundant and locally-available agricultural wastes containing high carbohydrate content, which serve as fermentable substrate for microorganisms (Bezalwar *et al*., 2013). The proximate analysis of the OA substrate showed that it contained 57.15% carbohydrate, 27.67% crude fibre, 4.69% crude protein, 0.62% lipids, 7.46% moisture and 2.41% ash (Table 1). This is within the range reported by other workers which have shown OA to contain between 40-64 % carbohydrate, 2-9% protein, 17-35% crude fibre and 0.85 – 13.4% ash (Oikeh *et al.*, 2013; M'Hiri *et* al., 2015; Taha *et al*., 2015; Hassan *et al.*, 2016; Romelle *et al*., 2016). The high carbohydrate content constituted a good carbon source for the growth of the fermenting organisms. In addition, the amount of carbohydrate relative to protein gave a high C/N ratio which is recommended for a good GA production (Kumar and Lonsane, 1989). The production of gibberellins starts during fermentation when nitrogen is depleted in the medium, and continues when enough carbon is available in the substrate (Escamilla *et al*., 2000). Nitrogen repression is a well-known regulatory principle for secondary metabolite formation (Munoz and Agosin, 1993). A good substrate should provide sufficient nutrients for the initial mycelial growth of the fermenting fungi in a nitrogen-limited but balanced medium (Rodrigues *et al*, 2009). This substrate with 57.15% carbohydrate and 4.69% protein fits this criterion. The presence of lipids in the OA is beneficial. Kawanabe *et al*. (1983) and Tudzynski (1999) reported that the biosynthesis of GA is based on acetate and follows the isoprenoid pathway. Therefore, plant oils are inert for catabolite repression. They also provide a pool of acetyl CoA, and may yield precursors for GA biosynthesis.

The highest GA yield by *F. moniliforme* and *A. niger* on OA before optimization was 5.53 g/L and 6.30 g/L respectively with *A. niger* showing higher productivity (Table 2). These differences in yields were statistically significant (p<0.05). This yield was higher than 680 mg/L reported by Muddapur *et al.* (2015) using *Fusarium* sp.; 2.86 g/L by *G. fujikuroi* (Escamilla *et al*., 2000); 0.7 g/L by *G. fujikuroi* (Lale *et al*., 2006); 1.82 g/L by *F. moniliforme* (Kobomoje *et al*., 2013); 2.8g/L by *F. moniliforme* (Pastrana *et al*., 1993); and 460.06 mg/L by *G. fujikuroi* reported by Cuali-Alvarez *et al*. (2011). However they were lower than 11.3 g/L by *F. moniliforme* (Bilkay *et al*., 2010) and 15 g/L and 32.8 g/L reported by Rangaswamy (2012) and Omojasola and Benu (2016) on *Jatropha* seedcake using *F. moniliforme* and *A. terreus* respectively. The various differences in the GA yields may be attributed to the differences in the conditions of fermentation, substrates and fermenting organisms. *F. moniliforme* and *A. niger* that were used for fermentation in this study are highly cellulolytic (Dashtban *et al*., 2009) and efficient in the utilization of the cellulosic substrate. Physiological factors often determine the outcome of the fermentation process, and may influence the yield of GA (Kahlon and Malhotra, 1986; Karakoc and Aksoz, 2006).

In studying the effect of time on the GA yield, it was observed that GA production commenced on day one of fermentation (Figure 1). This correlates with the observation of Ates *et al*. (2006) and Lale and Gadre (2010) who also recorded GA yields within the first twenty-four hours of fermentation. However, it is contrary to some findings that GA was recorded about forty-six hours after the commencement of fermentation following nitrogen depletion in the medium (Escamilla *et al*., 2000; Rodrigues *et al*., 2009; Rios-Iribe *et al*., 2011). The early onset of GA production may be attributed to the small amounts of protein in the OA substrate leading to its speedy exhaustion (Shukla *et al.*, 2005; Sleem, 2013). GA production peaked on day six for *F. moniliforme* and day five for *A. niger* (Figure 1). Peak GA yields have reported between days 4-8 for *F. moniliforme* (Kumar and Lonsane, 1990; Meleigy and Khalaf, 2009; Rangaswamy, 2012; Omojasola and Benu, 2016) and days 6-12 for *A. niger* (Bilkay *et al*., 2010).

It was observed that the GA yield was highest at pH 5.0 and 5.5 for *F. moniliforme* and *A. niger* respectively (Figure 2). This agrees with the works of other researchers who also observed peaks in GA yields at similar pH ranges (Qian *et al*., 1994; Shukla *et al*., 2005; Bilkay *et al.*, 2010; Kobomoje *et al*., 2013). Borrow *et al*. (1964) observed that GA production decreased when pH was outside the range of 3.0-5.5 in a stirred culture. The pH is considered one of the most important factors on biomass and yield because of its great influence on the physiological activities of the fermenting organisms (Sleem, 2013).

Maximum GA yields were observed when 2% inoculum of both *F. moniliforme* and *A. niger* were used for the fermentations (Figure 3). However, statistical

comparisons of means showed no significant differences (p<0.05) between the peak yields obtained when 1.5% and 2% inoculum of *F. moniliforme*; and 1% and 1.5% inoculum of *A. niger* were used. The use of sufficient inoculum for fermentation purposes is important as inadequate inoculum may lead to reduction in biomass and GA production, while excessive inoculum can also lead to low yields resulting from overpopulation and subsequent competition for available nutrients by the fungi (Omojasola and Benu, 2016).

The highest GA yields were recorded at 3 g substrate concentration for both fungi (Figure 4). A balanced amount of substrate is necessary for a good GA production. GA is a secondary metabolite produced in the log/stationary phase of growth. Low glucose concentration (< 4%) is required for GA production and maintenance of biomass in the production phase (Kumar and Lonsane, 1989), meanwhile GA biosynthesis is suppressed by high amounts (>20%) of glucose (Bruckner, 1992).

Supplementation of the fermentation media with different concentrations of copper sulphate appeared to show negligible and no significant difference (p<0.05) in GA yield (Table 3). This observation differs from the report of Arakaki *et al.* (2011), who found improved biomass production in yeasts grown under submerged fermentation when $CuSO_4$ was incorporated. The inability of the supplement to increase GA production may be because it is not essential in the normal physiological activities of both *F. moniliforme* and *A. niger*, especially with respect to the production of GA.

After the optimization experiment, the peak production of GA by *F. moniliforme* and *A. niger,* was observed on day six of the fermentation (Table 4). This is in tandem with Meleigy and Khalaf (2009) and Omojasola and Benu (2016) who reported GA production by *F. moniliforme* and *A. niger* respectively to be optimum on day six of the fermentation. In contrast to the pre-optimized fermentations, *F. moniliforme* showed higher productivity. Generally, the optimization of fermentation conditions provided significantly (p<0.05) higher yields compared to pre-optimization. The optimized yields obtained on OA by *F. moniliforme* and *A. niger* were 9.39 g/L and 7.42 g/L respectively; corresponding to a 69.8% and 17.78% increase respectively. These results are consistent favorably with those of Ates *et al*. (2006) who reported GA yields 13.0 mg/100 mL and 16.0 mg/100 mL by *G. fujikuroi* and *A. niger* respectively, which increased to 17.5 mg/100 mL and 20.5 mg/100 mL respectively after the optimization using silicone oil. The % GA recovery from the fermentation medium was 5.6% equaling 56.0 mg of GA per g of OA substrate fermented.

5. Conclusion

The production of GA through submerged fermentation of OA by *Fusarium moniliforme* ATCC 10052 and *Aspergillus niger* CBS 513.88 is described in this study. The results indicate that orange albedo (OA) is a cheap and readily available substrate for the production of GA. Yields of GA produced by *F. moniliforme* and *A. niger* were 9.39 g/L and 7.42 g/L respectively. However, in the optimization experiments, yields of 15.97 g/L and 10.74 g/L were produced by *F. moniliforme* and *A. niger* respectively, which are among the highest reported in literature demonstrating that the OA substrate can be used for the efficient production of GA. This indicates that high yields are dependent on the use of appropriate physical and nutritional conditions during fermentation. It can be concluded that *Fusarium moniliforme* ATCC 10052 and *Aspergillus niger* CBS 513.88 can be employed on a large scale in the production of this valuable acid using the agro-waste of OA, which will also help reduce the environmental pollution.

References

Akter N, Islam MR, AbdulKarim M and Hossain T. 2014. Alleviation of drought stress in maize by exogenous application of gibberellic acid and cytokinin. *J Crop Sci Biotechnol,* **17(1)**: 41 – 48.

Alrashdi AMA, Al-Qurashi AD, Awad MA, Mohamed SA and Al-rashdi AA. 2017. Quality, antioxidant compounds, antioxidant capacity and enzymes activity of 'El-Bayadi' table grapes at harvest as affected by preharvest salicylic acid and gibberellic acid spray. *Sci Hort* **220**: 243 – 249.

Alvarenga R, Moraes JC, Auad AM, Coelho M and Nascimento AM. 2017. 1nduction of resistance of corn plants to *Spodoptera frugiperda* (J. E. Smith, 1797) (Lepidoptera: Noctuidae) by application of silicon and gibberellic acid. *Bull Entomol Res*, **107(4)**:1-7.

Ambawade MS and Pathade GR. 2015. Production of gibberellic acid by *Bacillus siamensis* BE 76 isolated from banana plant (*Musa* spp). *Int J Sci Res,* **4(7)**: 394 – 398.

AOAC. 1990. Official Methods of Analysis of the Association of Official Analytical Chemists (15thed.). Arlington, Virginia.

AOAC. 2002. Official Methods of Analysis of the Association of Official Analytical Chemists (17thed.) Volume I and II. Maryland.

Arakaki AH, Vandenberghe LP, Soccol VT, Masaki R, Filho EF, Gregório A and Soccol CR. 2011. Optimization of biomass production with copper bioaccumulation by yeasts in submerged fermentation. *Braz Arch Biol Technol,* **54(5)**: 1027-1034.

Ates S, Ozenir S and Gökdere M. 2006. Effect of silicone oil on gibberellic acid production by *Gibberella fujikuroi* and *Aspergillus niger. Appl Biochem Microbiol,* **42(5)**: 500–501.

Barani M, Akbari N and Ahmadi H. 2013. The effect of gibberellic acid (GA_3) on seed size and sprouting of potato tubers (*Solanum tuberosum* L.). *Afr J Agric Res*, **8(29)**: 3898-3903.

Berrios J, Illanes A and Aroca G. 2004. Spectrophotometric method for determining gibberellic acid in fermentation broths. *Biotechnol Lett,* **26(1)**: 67 – 70.

Betiku E, Emeko HA and Solomon BO. 2016. Fermentation parameter optimization of microbial oxalic acid production from cashew apple juice. *Heliyon,* **2(2)**:e00082.

Bezalwar P, Ashok VG, Harshal MS and Pranita AG. 2013. Production and optimization of citric acid by *Aspergillus niger* using fruit pulp waste. *Int J Curr Microbiol Appl Sci*, **2(10)**: 347-352.

Bilkay IS, Karakoc S, Aksoz N. 2010. Indole-3-acetic acid and gibberellic acid production in *Aspergillus niger*. *Turk J Biol*, **34**: 313-318.

Borrow A, Brown S, Jefferys EG, Kessell RHJ, Lloyd EC, Lloyd PB, Rothwell A, Rothwell B and Swait JC. 1964. The effect of varied temperature on the kinetics of metabolism of *Gibberella fujikuroi* in stirred culture. *Can J Microbiol*, **10**: 445 – 466.

Bradley RL. 2010. Moisture and total solids analysis. *In*: **Food Analysis**. Springer, US, pp 85 – 104.

Bruckner B. 1992. Regulation of gibberellins formation by the fungus *Gibberella fujikuroi. Ciba Foundation Symposium* **171**: 129-137.

Bruckner B and Blechschmidt D. 1991. The gibberellin fermentation. *Crit Rev Biotechnol*, **11(2)**: 163-192.

Chinedu SN, Eni AO, Adeniyi AI and Ayangbemi JA. 2010. Assessment of growth and cellulase production of wild-type microfungi isolated from Ota, Nigeria. *Asian J Plant Sci*, **9(3)**:118-125.

Cihangir N. 2002. Stimulation of the gibberellic acid synthesis by *Aspergillus niger* in submerged culture using a precursor. *World J Microbiol Biotechnol*, **18***:* 727–729.

Cuali-Alvarez I, Pavon-Romero SH and Colin-Cruz A. 2011. Production of gibberellic acid from *Gibberella fujikuroi* using municipal sewage sludge as a substrate. *Univ Sci*, **16(1)**: 51-62.

Dashtban M, Schraft H and Qin W. 2009. Fungal bioconversion of lignocellulosic residues: opportunities and perspectives. *Int J Biol Sci,* **5**: 578-595.

Da Silva AL, Rodrigues C, Costa JD, Machado MP, Penha RD, Biasi LA, Vandenberghe LP and Soccol CR. 2013. Gibberellic acid fermented extract obtained by solid-state-fermentation using citrus pulp by *Fusarium moniliforme*: Influence on *Lavandula angustifolia* Mill., cultivated *in vitro. Pak J Bot*, **45(6)**: 2057-2064.

Escamilla EM, Dendooven L, Magana IP, Parra R and De La Torre M. 2000. Optimization of gibberellic acid production by immobilized *Gibberella fujikuroi* mycelium in fluidized bioreactors. *J Biotechnol,* **76**: 147 – 155.

Gupta R and Chakrabarty SK. 2013. Gibberellic acid in plant: Still a mystery unresolved. *Plant Signal Behav,* **8(9)**: e25504.

Hassan AA, El- Gharabli MM, El-Desouky AI, Eltanahy HH and Abdelslam AM. 2016. Technological feasibility of preparing spaghetti enriched with some by-products of food industry in Egypt. *Ann Agric Sci*, **54(4)**: 853–864.

Kahlon SS and Mahlotra S. 1986. Production of gibberellic acid by fungal mycelium immobilized in sodium alginate *Enzyme Microb Technol*, **8**:613-616

Karacoç S and Aksöz N. 2006. Some optimal cultural parameters for gibberellic acid biosynthesis by *Pseudomonas* sp. *Turk J Biol,* **30**: 81 – 85.

Kareem SO and Rahman RA. 2013. Utilization of banana peels for citric acid production by *Aspergillus niger. ABJNA*, **4(4)**: 384-387.

Kawanabe Y, Yamane H, Murayama T, Takahashi N and Nakamura T. 1983. Identification of gibberellins A_3 in mycelia of *Neurospora crassa. Agric Biol Chem*, **47**: 1693-1694.

Kobomoje OS, Mohammed AO and Omojasola PF. 2013. The production of gibberellic acid from shea nut shell (*Vitellaria paradoxa)* using *Fusarium moniliforme. Asian J Plant Sci Res,* **3(2)**: 23-26.

Kumar S, Venkata DV and Pakshirajan K. 2011. Purification and characterization of

glutaminase-free L-Asparaginase from *Pectobacterium carotovorum* MTCC 1428. *Bioresourc Technol*, **102(2)**: 2077–2082.

Kumar PKR and Lonsane BK. 1989. Microbial production of gibberellins: state of the art. *Adv Appl Microbiol*, **34**: 29–139.

Kumar PKR and Lonsane BK. 1990. Solid state fermentation: physical and nutritional factors influencing gibberellic acid production. *Appl Microbiol Biotechnol*, **34**: 145-148.

Lale G, Jogdand VV and Gadre RV. 2006. Morphological mutants of *Gibberella fujikuroi* for enhanced production of gibberellic acid. *J Appl Microbiol,* **100**: 65-72.

Lale G and Gadre R. 2010. Enhanced production of gibberellin A_4 (GA_4) by a mutant of *Gibberella fujikuroi* in wheat gluten medium. *J Ind Microbiol Biotechnol*, **37**: 297–306.

Macmillan J. 2002. Occurrence of gibberellins in vascular plants, fungi, and bacteria. *J Plant Growth Regul,* **20**: 387-442.

Mai HTN, Lee KM and Choi SS. 2016. Enhanced oxalic acid production from corncob by a methanol-resistant strain of *Aspergillus niger* using semi solid-state fermentation. *Proc Biochem,* **51**:9-15.

Mander LN. 2003. Twenty years of gibberellin research. *Nat Prod Rep*, **20(1)**: 49-69.

Meleigy SA and Khalaf MA. 2009. Biosynthesis of gibberellic acid from milk permeate in repeated batch operation by a mutant *Fusarium moniliforme* cells immobilized on loofa sponge. *Bioresourc Technol*, **100**: 374–379.

M'Hiri N, Ioannru I, Ghoul M, Doudhrioua MN. 2015. Proximate chemical composition of orange peel and variation of phenols and antioxidant activity during convective air drying. *Agri & B io Tech,* **(9)**: 881-890.

Muddapur UM, Gadkari MV, Kulkarni SM, Sabannavar PG, Niyonzima FN and More SS. 2015. Isolation and characterization of gibberellic acid 3 producing *Fusarium* sp. from Belgaum agriculture land and its impact on green pea and rice growth promotion. *AJAPB,* **1(2)**: 1-9.

Munoz GA and Agosin E. 1993. Glutamine involvement in nitrogen control of gibberellic acid production in *Gibberella fujikuroi. Appl Environ Microbiol,* **5(2)**: 298-310.

Nandini S, Nandini KE and Krishna SS. 2014. Food and Agriculture Residue (FAR): A potential substrate for tannase and gallic acid production using competent microbes. *J Bioprocess Biotech,* **5**: 193.

O'Donnell K, Cigelnik E and Nirenberg HI. 1998. Molecular systematics and phylogeography of the *Gibberella fujikuroi* species complex. *Mycologia,* **90**: 465–93.

Oikeh EI, Oriakhi K and Omoregie ES. 2013. Proximate analysis and phytochemical screening of *Citrus sinensis* fruit wastes. *The Bioscientist,* **1(2)**: 164 – 170.

Omojasola PF, Okun HA, Oladoye CO, Kayode RMO and Ahmed El-Imam A. 2014. Citric acid production by *Aspergillus niger* and *Trichoderma longibrachiatum* using *Ananas cosmosus* waste and *Saccharum officunarum* baggasse. *Nig J Biochem Mol Biol*, **29(2)**: 117-127.

Omojasola PF and Adeniran EA. 2014. The production of itaconic acid from sweet potato peel using *Aspergillus niger* and *Aspergillus terreus. Albanian J Agric Sci*, **13(4)**: 1-5.

Omojasola PF and Benu OE. 2016. Fermentative production of gibberellic acid from *Jatropha curcas* seed cake using A*spergillus niger* and *Aspergillus terreus. FUTA Journal of Research in Sciences,* **12(2)**: 242 – 251.

Pastrana LM, Gonzalez MP and Murado MA. 1993. Production of gibberellic acid from mussel processing wastes in submerged batch culture. *Bioresourc Technol,* **4(5)**: 213 – 221.

Qian X-M, du Preez JC and Kilian SG. 1994. Factors affecting gibberellic acid production by *Fusarium moniliforme* in solid-state cultivation on starch. *World J Microbiol Biotechnol*, **10**: 93-99.

Rachev RC, Pavlova-Rouseva R, Bojkova SV and Gancheva VK. 1993. Isolation of gibberellic acid produced by *Fusarium moniliforme*. *J Nat Prod*, **56(7)**: 1168-1170.

Rademacher W. 1994. Gibberellic formation in microorganisms. *Plant Growth Regulation*, **15**: 303-314.

Rademacher W 2016. Chemical regulators of gibberellin status and their application in plant production. *In*: **Annual Plant Reviews: The Gibberellins (Vol 49),** Hedden P and Thomas SG (Eds.), Wiley-Blackwell, UK, pp 359–404.

Rangaswamy V. 2012. Improved production of gibberellic acid by *Fusarium moniliforme*. *J Microbiol Res*, **2(3)**: 51-55.

Rios-Iribe EY, Flores-Cotera LB, Gonzalez-Chavira MM, Gonzalez-Alatorre G, Escamilla-Silva EM. 2011. Inductive effect produced by a mixture of carbon source in the production of gibberellic acid by *Gibberella fujikuroi. World J Microbiol Biotechnol*, **27**: 1499–1505.

Rivas B, Torrado A, Torre P, Converti A and Dominguez JM. 2008. Submerged citric acid fermentation on orange peel autohydrolysate. *J Agric Food Chem*, **56**: 2380-2387.

Rodrigues C, Vandenberghe LP, Teodoro J, Oss JF, Pandey A and Soccol CR. 2009. A new alternative to produce gibberellic acid by solid state fermentation. *Braz Arch Biol Technol*, **52(special)**:181-188.

Rodriguez-Fernandez DE, Rodriguez-Leon JA, de Carvalho JC, Sturm W, Soccol CR. 2011. The behaviour of kinetic parameters in production of pectinases and xylanase by solid state fermentation. *Bioresourc Technol*, **102**: 10657-10662.

Romelle FD, Ashwini RP, Ragu SM. 2016. Chemical composition of some selected fruit peels. *Eur J Food Sci Technol*, **4(4)** 12-21.

Santos EMG, Couto CMCM, Montenegro MCBSM, Neves MGPMS, Rebelo SLH, Cavaleiro JAS and Reis, BF. 2003. Ion-selective electrodes based on metallo-porphyrins for gibberellic acid determination in agricultural products. *Anal Bioanal Chem,* **375**: 511 – 516.

Shukla R, Chand S and Srivastava AK. 2005. Improvement of gibberellic acid production using a model based fed-batch cultivation of *Gibberella fujikuori*. *Process Biochem*, **40**: 2045 – 2050.

Sleem DAE. 2013. Studies on the bioproduction of gibberellic acid from fungi. PhD Thesis, Benha Univ, Egypt.

Subramaniyam R and Vimala R. 2012. Solid state and submerged fermentation for the production of bioactive substances: A comparative study. *Int J Sci Nat*, **3(3)**: 480-486.

Taha FT, Elsaadany SS, Abo-Eyta AM and Wahden KM. 2015. Proximate compositions, phytochemical constituents and antimicrobial activities of peels extracts of bitter orange and banana. *Zagazig J Agri Res.,* **42(6)**:

Torrado AM, Cortes S, Salgado JM, Max B, Rodriguez N, Bibbins B, Converti A and Dominguez JM. 2011. Citic acid production from orange peel wastes by solid state fermentation. *Braz J Microbiol*, **42(1)**: 394-409.

Tudzynski B. 1999. Biosynthesis of gibberellins in *G. fujikuroi* biomolecular aspects. *Appl Microbiol Biotechnol*, **52**: 298-310.

United States Department of Agriculture USDA. 2017. Citrus: World Markets and Trade. *Foreign Agricultural Service. July 2017*: 1-9. (Sept. 27, 2017).

Viniegra-Gonalez G, Favela-Torres E, Aguilar CN, Romero-Gomez SJ, Diaz-Godinez G and Augur C. 2003. Advantages of fungal enzyme production in solid state over liquid fermentation systems. *Biochem Eng J,* **13**: 157-167.

Yamaguchi C. 2008. Gibberellin metabolism and its regulation. .*Annu Rev Plant Biol,* **59**: 225-251.

Zhang B, Lu L and Xu G. 2015. Why solid-state fermentation is more advantageous over submerged fermentation in converting high concentration of glycerol into Monacolin K by *Monascus purpureus* 9901: A mechanistic study. *J Biotechnol*, **206**: 60-65.

Zhou IM, Ge XY and Zhang WG. 2011. Improvement of polygalacturonase production at high temperature by mixed culture of *Aspergillus niger* and *Saccharomyces cerevisae Bioresourc Technol*, **102**:10085-10088.

JJBS

Jordan Journal of Biological Sciences

Volume 11, Number 2, June .2018
ISSN 1995-6673
Pages 195 – 200

The Insecticidal Efficacy of the Extracts of *Piper nigrum* (Black Pepper) and *Curcuma longa* (Turmeric) in the Control of *Anopheles gambiae* Giles (Dip., Culicidae)

Kehinde A. Kemabonta[1,*] Olusola I. Adediran [2] and Kafayat O. Ajelara[3]

[1,2]Department of Zoology, Faculty of Science, [3]Department of Zoology, and Environmental Biology, Faculty of Science, Lagos State University, Lagos. Nigeria

Received August 6, 2017; Revised October 28, 2017; Accepted November 18, 2017

Abstract

The use of botanicals as alternatives to synthetic insecticides offers a more environmentally friendly method of insect control. The current study evaluated the toxic effects of the essential oils, aqueous and methanolic extracts of *Piper nigrum* (black pepper) and *Curcuma longa* (turmeric) extracts on the larvae, pupae and adults of *Anopheles gambiae*. The moulded insecticidal coils and essential oils of the plants were used to determine adults' repellence in the laboratory. The essential oils were most effective against *A. gambiae* larvae showing 100% mortality for both *P. nigrum* and *C. longa* with LC_{50} values of 15 and 149 ppm for *P. nigrum* and *C. longa*, respectively. The methanolic extracts had LC_{50} value of 27 and 214 ppm for *P. nigrum* and *C. longa*, respectively. Maximum (100%) concentration of the essential oils of *C. longa* and *P. nigrum* oils gave 95.7% and 95.3% protection, respectively. The insecticidal coil (100% concentration) of *P. nigrum* and *C. longa*, showed 76.7% and 70% knockdown action on the adult mosquitoes respectively. Therefore, *P. nigrum* and *C. longa* can serve as repellents against *A. gambiae*, and can be used in integrated vector management control programs.

Key words: *Piper nigrum* extracts, *Curcuma longa* extracts, Botanicals, *Anopheles gambiae*, Plant extracts, Insecticidal coils.

1. Introduction

Malaria, the leading cause of morbidity and mortality in Nigeria, causes about 1-2 million deaths (mostly amongst pregnant women and children under five years) annually in Africa (Breman, 2001; Muriu *et al*., 2013). In south-eastern Nigeria, *Anopheles gambiae* Giles (Dip., Culicidae) is the major malaria transmitting vector, followed by *A. funestus* (Uneke and Ibeh, 2009).

Vector control is an essential component of malaria prevention. Such control has been proven to successfully reduce or interrupt malaria transmission when coverage is sufficiently high (Innocent *et al*., 2014). The two cores, broadly applicable measures for malaria vector control are long-lasting insecticidal nets and indoor residual spraying (WHO, 2006). The burden of malaria is worsened by mosquito resistance to synthetic insecticides (Guinovart *et al*., 2006; Ranson and Lissenden, 2016), and malaria parasites' resistance to antimalarial drugs (Muriu *et al*., 2013; Raj *et al*., 2014; Ashley *et al*., 2014)

The problem of high cost and development of resistance in many mosquito species to several synthetic insecticides has revived interest in the use of botanicals as insecticides (Grainge and Ahmed, 1988). These bioactive chemicals may act as insecticides, antifeedants, moulting hormones, oviposition deterrents, repellents, juvenile hormone mimics, growth inhibitors, antimoulting hormones, and attractants (Adewoyin *et al*., 2006).

Botanicals as an alternative to synthetic insecticides in controlling mosquitoes, offer a more environmentally friendly method of insect control (Irungu and Mwangi, 1995). Apart from being of low mammalian toxicity and degradability, most insects find it difficult to resist the toxic effects of botanicals. Various plant species have been exploited, throughout the world, to control the mosquito populations (Muthukrishnan *et al*., 1997). *Piper nigrum* L. (black pepper) of the family Piperaceae, often called the 'King of Spices', is a universal table condiment used to flavour all types of cuisines worldwide (Mathew *et al*., 2001). The spicy taste can be attributed mainly to the presence of the compound Piperine which is a pungent alkaloid (Tripathi *et al*., 1996) that enhances the bioavailability of various structurally and therapeutically diverse drugs (Khajuria *et al*., 2002). It also contains small amounts of safrol, pinene, sabinene, limonene, caryophyllene and linaonol compounds. Black pepper is also an important traditional medicine used to treat asthma, chronic indigestion, colon toxins, obesity, sinus, congestion and fever, intermittent fever, cold extremities, colic, gastric ailments and diarrhoea (Balasubramanian *et al.*, 2016). The plant has been credited with interesting pesticidal properties against insect pests (Maenthaisong *et al*., 2014; Custódio *et al*., 2016; Samuel *et al*., 2016).

* Corresponding author. e-mail: kennykemabonta@yahoo.com.

Curcuma longa (Turmeric), of the family Zingiberaceae, is one of most essential spices used all over the world particularly in the countries of the east (Ravindran, 2007). It is a deep yellow-to-orange powder that comes from the underground stems of the tropical perennial herb. Primarily, phenolic compounds and terpenoids have been identified from this spice (Li *et al*., 2011). The plant has been credited with interesting pesticidal properties against insects and fungi of agricultural significance, including repellent properties against mosquito species (Ali *et al*., 2015; Tisgratog *et al*., 2016).

The aim of this study is to look for a more environmental friendly way, and a cheap, and readily-available effective method to control *A. gambiae.* This research was, therefore, carried out to investigate the efficacy of the essential oils, crude aqueous extracts and methanolic extracts of *P. nigrum* and *C. longa* against larvae, pupae and adult stages of *A. gambiae.*

2. Materials and Methods

2.1. Collection and Preparation of Test Materials

The seeds of *P. nigrum* and fresh rhizomes of *C. longa* were purchased from local markets in Lagos Nigeria. The *P. nigrum* seeds were dried for ten days at room temperature, then powdered using a Binatone blender. The *C. longa* rhizomes were cut into smaller pieces and dried for ten days before being grinded with blenders. The powders were sieved using a mesh size of 0.05 mm^2 to get a fine powder stored in amber bottles on the laboratory shelf for later use.

2.2. Preparation of Extracts

Crude aqueous extracts were obtained by separately dissolving fifty g of *P. nigrum* and *C. longa* powder in 500 ml of distilled water leaving them for twenty-eight hours. The liquid extracts were carefully collected by manually squeezing the soaked powder out. Then they were kept in amber bottles in a refrigerator to retain the potency of its active components for future use. The same method was adopted in the preparation of methanolic extract only that methanol was used in the place of distilled water. Essential oil was obtained by the hydro-distillation process using the Clevenger apparatus of the Department of Chemistry at Lagos State University in Ojo Lagos, Nigeria. Three replicates of 200 g of powdered *P. nigrum* and *C. longa* were separately introduced into the apparatus. The concentration of the *P. nigrum* was 1.75%, while that of the *C. longa* was 1.50%. The process lasted for three hours each. Hexane was added in the process in order to make the oil insoluble in water, and to demarcate the water from the oil, and avoid evaporation. Oil was collected in amber bottles and kept in the refrigerator to retain its potency until needed. One ml of essential oil was dissolved in 0.3 ml of hexane, which was mixed in 100 ml of distilled water to form a stock solution from which serial dilutions were made. The larvae, pupae and adult mosquitoes were obtained from a laboratory culture maintained in the Department of Zoology and Environmental Biology of Lagos State University, Lagos.

2.3. Bioassays and Test Procedures

Ten larvae of the second and third instar of *A. gambiae* were introduced. Mortality rate was recorded after twenty-four hours. 50 ppm to 450 ppm serial dilutions (50, 100, 150, 200, 250, 300, 350, 400 and 450 ppm) were used for turmeric, while 100 mL of distilled water served as a control. Three replicates were set for each concentration. The same procedure was carried out using a methanolic extract.

A larva was classified dead if it did not move when gently touched with the tip of moistened camel hairbrush, or when found lying immobile at the bottom of the container or not showing the characteristic diving reaction when water is disturbed. Same procedures were used for essential oils of the test plants on *A. gambiae* larvae.

Ten *A. gambiae* pupae were introduced into a transparent plastic cup containing 100 mL of serial dilution made from stock solution of aqueous extracts at various concentrations ranging from 10 ppm to 90 ppm for black pepper, and from 50 ppm to 450 ppm for turmeric extracts. Mortality rates were recorded after twenty-four hours using slandered units. Ten pupae in 100 mL of distilled water in plastic cup served as control. Three replicates were also set for each concentration. The same procedure was carried out using a methanolic extract.

2.4. Preparation and Tests Using Insecticidal Coils

Ground fine plant powder and wood shavings were used to mould coils of various concentrations ranging from 0% (control) to 100% (absolute plant powder mould). The insecticidal coil is a smoke test used to check for the repellent action of burnt plant extracts on mosquitoes. Coils moulded into different concentrations were burnt in a rectangular cage (measuring 59×44×45) containing twenty adult mosquitoes. Three replicates were set for each concentration with the wood shavings alone serving as the control. The dried coil was lit and the number of dead mosquitoes or those knocked down were counted and recorded for thirty minutes.

2.5. Data Analysis

The data of different treatments and different concentration of the same treatment were statistically analyzed using Finney (1947) formula to correct for mortality. The LC50, LC99, parameter estimates and chi-square values were calculated by using probit analysis (Finney, 1947). Data obtained from larval mortality and repellent activity were subjected to the analysis of variance (ANOVA). The statistical software SPSS was used for data analysis. Means were compared using Duncan's Multiple Range Test at 0.05% level of significance.

3. Results and Discussion

3.1. The Effect of Aqueous, Methanolic Extracts and Essential Oil on Anopheles gambiae Larvae

The concentration of the extracts was positively correlated with the mortality of the larvae. The correlation was 0.84, 0.94 and 0.96 for essential oils, aqueous and methanolic extracts of *P. nigrum*, respectively (Figure 1). As for the *C. longa*, the correlation was 0.94, 0.97 and 0.98, respectively (Figure 2). After a twenty-four-hour treatment, using 90 ppm concentration of *P. nigrum*, the mortality rate of *A. gambiae* larvae was 100%, 90% and

83.3% using essential oil, methanol and aqueous extracts, respectively (Table 1). On the other hand, 450 ppm concentration of *C. longa* gave 100, 86.7% and 76.7% mortality rates, respectively (Table 2). There was no mortality in the control treatment after twenty-four hours.

In almost all the concentrations of the botanicals used, the essential oil was significantly more toxic and effective on the mortality of larvae than the methanolic extract, which was significantly (P<0.05) more toxic than the aqueous extract (Tables 1 and 2). Moreover, 90 ppm of the essential oils of *P. nigrum* gave 100% larval mortality after twenty-four hours, while 450 ppm of *C. longa* gave the same mortality rate using the same time. The LC_{50} for the essential oils of *P. nigrum* on *A. gambiae* larvae was 15.4 ppm, while that for *C. longa* was 148 ppm. Amer and Mehlhorn (2006) reported larvicidal activity of *P. nigrum* with LC_{50} values between 10 and 105 ppm against *A. stephensi* Liston, *Ae. aegypti* and *Culex quinquefasciatus* after a twenty four-hour exposure. On the other hand, Kalaivani *et al*. (2012) found the LC_{50} of *C. longa* extract to be effective against the larvae of *Ae. aegypti* at 115.6 ppm. Similar results by Srivastava *et al*. (2003) of the aqueous and methanolic extract of *Nerium indium* lattices against the *C. quinquefasciatus* showed that different dilutions of the lattices delay the post embryonic development of *Culex* larvae, and was 1.8 times more toxic than the aqueous extract. Moreover, Akinneye and Afolabi (2014) showed that the aqueous extract of *Cleisthopholis patens* was relatively ineffective against the larvae of *Anopheles gambiae*. They concluded that the ineffectiveness of aqueous extract may be attributed to the fact that water was used for the extraction, and water is a polar solvent. Fafioye *et al*. (2004) reported that the ethanolic oil extracts of *Parkia biglobosa* and *Raphia vinifera* were more potent against the juvenile of *Clarias gariepinus* than the aqueous forms. This is due to the polarity, volatility and ethanol's power to dissolve more of the active ingredients.

The crude extracts and essential oils of plant species have a complex mix of chemical elements. These secondary metabolites have been used empirically in vector control and causal agents of the disease (Granados-Echegoyen *et al*., 2014). The developmental stage of the mosquito species determines the effectiveness of the plant extract (Ebe *et al*., 2015). The mosquito larvae stage is the most susceptible to any treatment, and is restricted to the common aquatic habitats (Bisset *et al*., 2014)

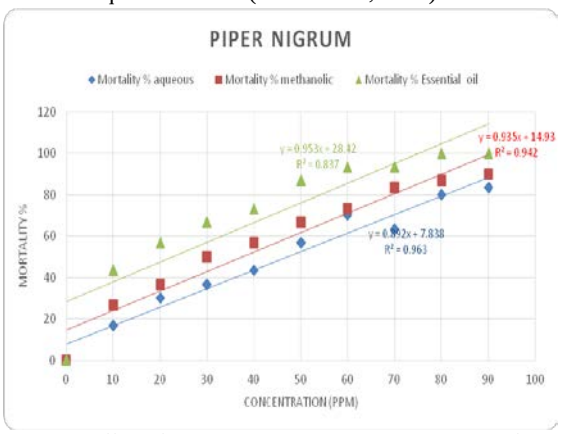


Figure 1. Effect of aqueous, methanolic and essential oils of *Piper nigrum* on larvae of *Anopheles gambiae* after 24 hours.

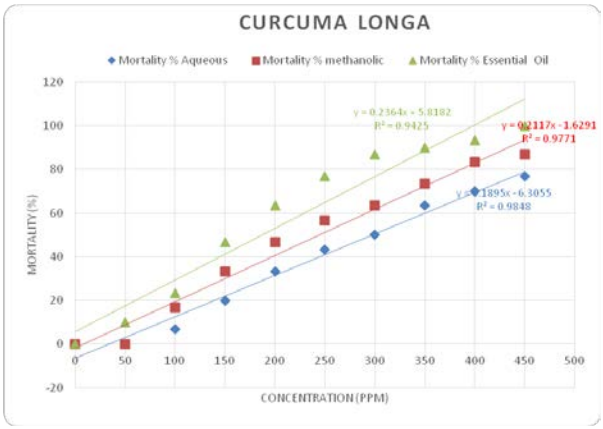


Figure 2. Effect of aqueous, methanolic and essential oils of *Curcuma longa* on *Anopheles gambiae* after 24 hours of treatment.

Table 1. Effect of the different extracts of *Piper nigrum (*Black pepper) on *Anopheles gambiae* larvae after 24hours of treatment.

Conc. (ppm)	Mean mortality			Mortality %		
	Aqueous	Methanolic	Essential oil	Aqueous	Methanolic	Essential oil
Control	0.00±0.00 ^{a}	0.00±0.00 ^{a}	0.00±0.00 ^{a}	0	0	0
10	1.67±0.58^{a}	2.67±0.58^{b}	4.33±0.58^{c}	16.7	26.7	43.3
20	3.00±0.00^{a}	3.67±0.58^{a}	5.67±0.58^{b}	30	36.7	56.7
30	3.67±0.58^{b}	5.00±0.00^{b}	6.67±0.58^{c}	36.7	50	66.7
40	4.33±0.58^{a}	5.67±0.58^{b}	7.33±0.58^{c}	43.3	56.7	73.3
50	5.67±0.58^{a}	6.67±0.58^{b}	8.67±0.58^{c}	56.7	66.7	86.7
60	7.00±1.00^{a}	7.33±0.58^{a}	9.33±2.15^{b}	70	73.3	93.3
70	7.33±0.58^{a}	8.33±0.58^{b}	9.33±1.15^{c}	63.3	83.3	93.3
80	8.00±1.00^{a}	8.67±0.58^{a}	10.00±0.00^{b}	80	86.7	100
90	8.33±1.15^{a}	9.00±1.00^{b}	10.00±0.00^{c}	83.3	90	100

A total of three replicates for each concentration and 10 larvae in each replicate were used

Mean mortality with different superscripts in the same row are significantly different from each other at P<0.05.

Table 2. Effect of the different extracts of *Curcuma longa* (turmeric) on *Anopheles gambiae* larvae after 24hours of treatment.

Conc. (ppm)	Mean mortality			Mortality %		
	Aqueous	Methanolic	Essential Oil	Aqueous	Methanolic	Essential Oil
0	0.00±0.00 [a]	0.00±0.00 [a]	0.00±0.00 [a]	0.0	0.0	0.0
50	0.00±0.00[a]	0.00±0.00[a]	1.00±1.00 [a]	0.0	0.0	10.0
100	0.67±0.58[a]	1.67±0.58[b]	2.33±0.58 [c]	6.7	16.7	23.3
150	2.00±0.00[a]	3.33±0.58[b]	4.67±0.58 [c]	20.0	33.3	46.7
200	3.33±1.53[a]	4.67±0.58[b]	6.33±1.53 [c]	33.3	46.7	63.3
250	4.33±0.58[a]	5.67±1.15[b]	7.66±1.15 [c]	43.3	56.7	76.7
300	5.00±1.00[a]	6.33±1.53 [b]	8.67±1.53 [c]	50.0	63.3	86.7
350	6.33±1.15[a]	7.67±0.58 [b]	9.00±1.00 [c]	63.3	73.3	90.0
400	7.00±1.00[a]	8.33±1.15 [b]	9.33 ±1.15 [c]	70.0	83.3	93.3
450	7.67 ±0.58[a]	8.67±1.53 [b]	10.00±0.00 [c]	76.7	86.7	100.0

A total of three replicates for each concentration and 10 larvae per replicate

Mean mortality with different superscripts in the same row are significantly different from each other at $P<0.05$.

3.2. The Effects of Extracts on Anopheles gambiae Pupae

There was no mortality observed in the pupae test in both aqueous and methanolic extracts. After a forty-eight-hour treatment, most pupae had metamorphosed to adult in both *P. nigrum* and *C. longa* treatments. Murty *et al*. (1997) reported that when treated with a leaf extract of *Polyalthia longifolia* adults of *Culex quinquefasciatus* emerged deformed from pupae being caught in the outer shell of the insect. However, Candido *et al*. (2013) evaluated the effects of extracts from *Cnidosculos phyllacanthus, Ricinus communis,* and *Coutarea hexandra* on the developmental periods of *A. aegypti* larvae and pupae. They reported that the promising effects of these products on the pupal stage of *A. aegypti* are related to the morphological differences between the pupae and larvae, indicating that the mode of action of these products occurs through contact or choking, but not swallowing, because the pupal stage does not involve ingestion.

3.3. The Effect of Insecticidal Coils from Extracts on Adult Anopheles gambiae

The insecticidal coil repellency increased with the increase in the concentration of extracts and time of exposure. *A. gambiae* adult mortality rate of 76.7% and 70% was observed with *P. nigrum* and *C. longa* plants respectively. The LC_{50} and LC_{95} for *P. nigrum* coils were 52 and 499 while that for *C. longa*, was 63 and 468, respectively (Table 4). Similar results were obtained by Pavitha and Poornima, (2014) on the repellent potential of *Tagetes erecta* and *Callistemon brachyandrus* against *Anopheles stephensi*, *Culex infulus* and *Aedes Aegypti*. This is in accordance with Pavitha and Poornima (2014) who found that the repellent activity test for the cream formulation showed 89.87%, 87.5% and 90% protection while the smoke toxicity test for the incense coil showed 66.25%, 70% and 67.5% protection against *An. stephensi*, *Cx. infulus* and *Ae. aegypti*, respectively.

3.4. Toxicity Tests

The LC_{50} of the *P. nigrum* for the larvae of *A. gambiae* was the least and highest in essential oils (15ppm), and the aqueous extracts (52.4 ppm), respectively. The same trend was observed with *C. longa.* It was 148 ppm and 276 ppm for the larvae of *A. gambiae*, respectively. Anyaele *et al*. (2002) found that the methanolic extract *Piper guineense* (Schum) showed high toxicity on the third instar larvae of *Aedes aegypti* with LC_{50} of 1.7±0.84g/mL and had a mortality rate of 77% of the larvae. The result was also in agreement with the study of Karunamoorthi *et al*. (2014) on the mosquito repellent activity of the essential oil of *Juniperus procera* against *Anopheles arabiensis* at 1 and 5 mg/ cm^2 concentrations. The result showed repellency and protection (80.60% in 311 min) against *A. arabiensis*.

Table 4. Toxicity of *Piper nigrum* and Curcuma longa extracts on *Anopheles gambiae*.

	LC_{50} (95% C.L)	LC_{95}(95% C.L)	SLOPE±S.E	PROBIT LINE EQUATION
Piper nigrum				
Aqueous extract on larvae	36.9 (30.08-43.89)	222.9 (150.1-441.9)	2.10±0.30	Y=0.89x+7.8
Methanolic extract on larvae	26.8 (20.49-32.59)	177.7 (122.4-337.7)	2.00±0.29	Y=0.94x+14.9
Essential oil on larvae	15.4 (10.85-19.51)	79.5 (61.5-118.5)	2.31±0.32	Y=0.95x+28.4
Coil on adult mosquitoes	52.4 (45.37-61.07)	499.1 (317.6-1013.8)	1.68±0.19	Y=0.43x+4.1
Curcuma longa				
Aqueous extract on larvae	276.4 (246.9-213.6)	827.5 (640.9-1255.2)	3.45±0.45	Y=0.19x-6.3
Methanolic extract on larvae	214.1 (188.1-240.9)	659.5 (528.8-925.1)	3.38±0.40	Y=0.21x-1.63
Essential oil on larvae	148.5 (127.2-168.6)	441.5 (368.4-571.8)	3.48±0.38	Y=0.24x+5.8
Coil on adult mosquitoes	63.2 (53.9-74.7)	468.1(299.8-984.4)	1.89±0.25	Y=0.41x+3.0

4. Conclusion

The present study showed the acute toxicity and repellency of *P. nigrum* and *C. longa* against larvae and adult *A. gambiae* at varied concentrations. Extracts of *P. nigrum* and *C. longa* have shown strong toxicity and repellent activities against *A. gambiae* larvae and adults. Since these botanicals safe, cheap, abundant, and biodegradable, they can be used in the control of malaria vectors in rural areas.

More studies should, however, be carried out especially on *P. nigrun* that has a lower LC_{50} to discover, identify, and isolate the insecticidal compounds that can control the malaria vector. The government should encourage, empower and establish research institutes, give financial support for further research into the bioactivity of these plants to be able to formulate insecticidal coils that can be used to repel and prevent mosquito bites, which may eventually reduce the use of synthetic insecticides.

5. References

Adewoyin FB, Odaibo AB and Adewunmi CO. 2006. Mosquito repellent activity of *Piper guineense* and *Xylopia aethiopica* fruits oils on *Aedes aegypti*. *Afr Trad J CAM*, **3(2)**: 79-83.

Akinneye, JO and Afolabi, OJ. 2014. Toxicity and fumigant effect of powder and oil extracts of *Cleisthopholis patens* (Benth) against larvae and adults *Anopheleles* mosquito. *J. Mosq Res.*, ***4*(11)**: 1-6.

Ali A, Wang YH and Khan IA. 2015. Larvicidal and biting deterrent activity of essential oils of *Curcuma longa*, Ar-turmerone, and Curcuminoids against *Aedes aegypti* and *Anopheles quadrimaculatus* (Culicidae: Diptera). *J Med Ent.*, **52(5)**: 979-986.

Amer A and Mehlhorn H. 2006. Larvicidal effects of various essential oils against *Aedes*, *Anopheles* and *Culex* larvae (Diptera, Culucidae). *Parasitol Res.*, **99**: 466-472.

Anyaele O, Amusan AS, Okorie TG and Oke OA. 2002. Toxicity of methanolic extract of *P. guineense* Schum and Thonn seed oil to larvae of *Aedes aegypti*. *Nig J Ent.*, **15**: 15-21.

Ashley EA, Dhorda M, Fairhurst RM, Amaratunga C, Lim P, Suon S, Sreng S, Anderson JM, Mao S, Sam B and Sopha C. 2014. Spread of artemisinin resistance in *Plasmodium falciparum* malaria. *New England J Med.*, **371(5)**: 411-423.

Balasubramanian, S, Roselin P, Singh, KK, Zachariah, J and Saxena, SN 2016. Postharvest processing and benefits of black pepper, coriander, cinnamon, fenugreek, and turmeric spices. *Crit Rev Food Sci and Nutri.*, ***56*(10)**: 1585-1607.

Bisset JA., Rodríguez MM, French L, Severson DW, Gutiérrez G, Hurtado D and Fuentes I. 2014. Insecticide resistance and metabolic mechanisms involved in larval and adult stages of *Aedes aegypti* insecticide-resistant reference strains from Cuba. *J Am Mosq Cont Ass.*, ***30*(4)**: 298-304.

Breman J. 2001. The ears of the hippopotamus: manifestations, determinants and malaria burden. *Am J Trop Med Hyg.*, **64(1–2)**: 1-11.

Candido LP, Cavalcanti MT and Beserra EB. (2013). Bioactivity of plant extracts on the larval and pupal stages of *Aedes aegypti* (Diptera Culucidae). *Revis da Soc. Brasil. de Med. Trop.*, **4**6(4): 420-425.

Custódio KM, Oliveira JGD, Moterle D, Zepon KM, Prophiro JS and Kanis LA. 2016. A biodegradable device for the controlled release of *Piper nigrum* (Piperaceae) standardized extract to control *Aedes aegypti* (Diptera, Culicidae) larvae. *Revista da Sociedade Brasil. de Med. Trop.*, **49(6)**: 687-692.

Ebe TE, Mgbemena IC, Njoku-Tony RF, Njoku JD and Emereibeole E. 2015. Comparative analysis of petroleum ether and aqueous extracts of neem leaf and neem stem on different stages of *Anopheles gambiae*. *J. of Nat. Sci. Res.*, **5(12)**: 95-98.

Fafioye OO, Adebisi AA and Fayode SO. 2004. Toxicity of *Parkia biglobosa* and *Raphia vinifera* extracts on *Clarias gariepinus* juveniles. *Afr. J. Biotech*., **3(10)**: 627-630

Finney D. 1947. The Estimation from Individual Records of the Relationship Between Dose and Quantal Response. *Biometrika*, **34 (3/4)**: 320-334.

Grainge M and Ahmed S. 1988. **Handbook of Plants with Pest Control Properties**. John Wiley and Sons, New York, pp 41 and 470.

Granados-Echegoyen C, Pérez-Pacheco R., Soto-Hernández M., Ruiz-Vega J, Lagunez-Rivera L, Alonso-Hernandez N and Gato-Armas R. (2014). Inhibition of the growth and development of mosquito larvae of *Culex quinquefasciatus* (Diptera: Culicidae) treated with extract from leaves of *Pseudocalymma alliaceum* (Bignonaceae). *Asian Pac J of Trop Med*., **7(8)**: 594-601.

Guinovart C, Navia MM, Tanner M and Alonso PL. 2006. Malaria: burden of disease. *Currt Mol Med.*, **6(2**):137-140

.Innocent E, Hassanali A, Kisinza WN, Mutalemwa PP, Magesa S and Kayombo E. 2014. Anti-mosquito plants as an alternative or incremental method for malaria vector control among rural communities of Bagamoyo District, Tanzania. *J Ethnobiol Ethnomed.*, **10(1)**: 56.

Irungu LW and Mwangi RW. 1995. Effects of a biologically active fraction from *Melia volkensii* on *Culex quinquefasciatus*. *Inst Appl Sci*, **2:** 159-162.

Kalaivani K, Senthil-Nathan S and Murugesan AG. 2012. Biological activity of selected Lamiaceae and Zingiberaceae plant essential oils against the dengue vector *Aedes aegypti* L. (Diptera: Culicidae). *Parasitol Res.*, **110**: 1261–1268

Karunamoorthi K., Girmay A and Fekadu S. 2014. Larvicidal efficacy of Ethiopian ethnomedicinal plant *Juniperus procera* essential oil against Afrotropical malaria vector *Anopheles arabiensis* (Diptera: Culicidae). *Asian Pac J Trop Biomed.*, **4**: S99-S106.

Khajuria A, Thusu N and Zutshi U. 2002. Piperine modulates permeability characteristics of intestine by inducing alterations in membrane dynamics: influence on brush border membrane fluidity, ultra-structure and enzyme kinetics. *Phytomed*., **9(3)**: 224-231.

Li SY, Yuan W, Deng GR and Wang P. 2011. Chemical composition and product quality control of turmeric (*Curcuma longa* L.). *Pharm Crops*, **2**: 28-54.

Maenthaisong R, Chaiyakunapruk N, Tiyaboonchai W, Tawatsin A, Rojanawiwat A and Thavara U. 2014. Efficacy and safety of topical Trikatu preparation in relieving mosquito bite reactions: A randomized controlled trial. *Comp. Therap Med*., **22(1)**: 34-39.

Mathew PJ, Mathew PM and Kumar V. 2001. Graph clustering of *Piper nigrum* L. (Black pepper). *Euphytica*, **118**: 257-264.

Muriu SM, Coulson T, Mbogo CM and Godfray HCJ. 2013, Larval density dependence in *Anopheles gambiae* s.s., the major African vector of malaria. *J Anim Ecol.*, **82**: 166-174.

Murty US., Sriram K and Jamil K. 1997. Effect of leaf extract of *Polyalthia longifolia* (Family: Annonaceae) on mosquito larvae and puape of *Culex quinquefasciatus* (Diptera: Culicidae) Say of different habitats. *Intern Pest Cont.*, **39**: 52-53.

Muthukrishnan J, Pushpalatha E and Kasthuribhai A. 1997. Biological effects of four plant extracts on *Culex quinquefasciatus* larval stages. *Insect Sci Appl.*, **17:** 389-394.

Pavitha P and Poornima S. 2014. Repellent potential of *Tagetes erecta* L. and C*allistemon brachyandrus* Lindl. against mosquito larvae for formulation of herbal repellent compounds. *Intern J Innov Res Sci Eng Technol.*, **3(5)**:12030-12037.

Raj R, Biot C, Carrère-Kremer S, Kremer L, Guérardel Y, Gut J, Rosenthal PJ and Kumar V. 2014. 4-Aminoquinoline -β-Lactam conjugates: Synthesis, antimalarial, and anti -tubercular evaluation. *Chem Biol Drug Design,* **83(2)**:191-197.

Ranson H and Lissenden N. 2016. Insecticide resistance in African Anopheles mosquitoes: a worsening situation that needs urgent action to maintain malaria control. *Trends in Parasitol.*, **32(3):**187-196.

Ravindran PN. 2007. Turmeric: The golden spice of life. *In:* **Turmeric: The Genus Curcuma**, Ravindran PN, Nirmal Babu K, Sivaraman K (Eds.). CRC Press, Boca Raton, FL, USA, pp 1-14.

Samuel M, Oliver SV, Coetzee M and Brooke BD. 2016. The larvicidal effects of black pepper (*Piper nigrum* L.) and piperine against insecticide resistant and susceptible strains of Anopheles malaria vector mosquitoes. *Parasit Vec*., **9(1)**: 238 – 245

Srivastava VK., Singh SK., Rai M and Singh A. 2003. Toxicity of *Nerium indicum* and *Euphorbia royleana* lattices against *Culex quinquefasciatus* mosquito larvae. *Nig J Nat Prod Med*., **7**:61-4.

Tisgratog R, Sanguanpong U, Grieco JP, Ngoen-Kluan R and Chareonviriyaphap T. 2016. Plants traditionally used as mosquito repellents and the implication for their use in vector control. *Acta Tropica*, **157**:136-144.

Tripathi AK, Jain DC and Kumar S. 1996. Secondary metabolites and their biological and medical activities of Piper species plants. *J Med Aroma. Plant Sci*., **18:** 302-321.

Uneke C and Ibeh L. 2009. Impact of deforestation on malaria in South-eastern Nigeria: the epidemiology, socio-economic and ecological implication. *Internet J Third World Med*, **8**(1): 1-2.

WHO/CDS/NTD/WHOPES/GCDPP. 2006.1. Department of Control of neglected Tropical Diseases viii, 114 p

JJBS

Volume 11, Number 2,June 2018
ISSN 1995-6673
Pages 201 - 207

Jordan Journal of Biological Sciences

Genetic Diversity and Structure Analysis of Pea Grown in Iraq Using Microsatellite Markers

Nawroz A. R. Tahir[1,*], Djshwar D. Lateef[2], Dlshad A. Omer[3], Shadia H. S. Kareem[2], Dastan A. Ahmad[2] and Lanja H. Khal[1]

[1]*Horticulture Department;*[2]*Crop Science Department, College of Agricultural Sciences, University of Sulaimani ;* [3] *Ministry of Agriculture, General Directorate of Agriculture, Sulaimani, Iraq*

Received October 12, 2017; Revised November 15, 2017; Accepted November 20, 2017

Abstract

The diversity and population structure of twenty-two genotypes of peas (*Pisum sativum L.*) grown in Iraq were estimated using microsatellite [simple sequence repeats (SSRs)]. Nineteen SSR primers generated sixty-eight polymorphic bands with an average of 3.789 polymorphic bands per primer. The highest number of polymorphic bands stated for the locus AA5 (eight alleles). Means of gene diversity, PIC, marker index, resolving power and Shannon index were 0.562, 0.513, 2.090, 2.703 and 0.833, respectively. Principal component analysis and hierarchical cluster analysis clustered the pea genotypes into three major clades. Genotypes G1 (ORP-2), G5 (ORP-11) and G6 (ORP-12) and G7 (ORP-13) were genetically the most distant from the other genotypes. Analysis of molecular variance (AMOVA) revealed that differences among the populations accounted for 14 % of the total variation, whereas difference within the population was 86 %. The population structure assay conceded that the genotypes were grouped into two evident subpopulations. Only three genotypes: G4 (ORP-10), G11 (Ns minima) and G17 (Karina) were considered to be admixture. The multi-locus F_{ST} analysis revealed strong differentiation within the populations and significant isolation by distance. The results of this study revealed that different origins of genotypes had played a remarkable role in shaping the current patterns of genetic variation among these populations, many of which serve as good candidates for conservation and breeding programs.

Keywords: Clustering, Genetic diversity, *Pisum sativum,* SSRs.

1. Introduction

Pea (*Pisum sativum L.*) was the basic model organism used in Mendel's discovery of the laws of inheritance, making it the basis of modern plant genetics. Nevertheless, subsequent progress in pea genomics has become last among other crop species. Pea is a diploid plant with chromosome number 2n = 14 (Smýkal *et al*., 2012), it represents a major pulse crop grown for its protein content. In different zones of the world, it is an essential component of agro-ecological cropping systems. Genotypes identification and the evaluation of genetic variation of populations are important in genotypes protection and breeding program (Smýkal *et al*., 2012). Global climate change and the new technologies make pea breeders conduct more effective methods of selection and take benefit of the large genetic diversity present in the *Pisum sativum* gene pool (Smýkal *et al*., 2012).

Geneticists and plant breeders have affirmed the need for additional development in capturing and harnessing genetic variability. Several approaches are accessible to assess the diversity of genotypes. Traditional morphological or biochemical markers are restricted and are not fully-reliable because of the influence of the environment. The markers have been superseded by DNA-based methods that generate fingerprints (Hollingsworth, 2006). On this basis, various studies have been so far conducted on peas employing Random Amplification of Polymorphic DNA (RAPD), Simple Sequence Repeats (SSRs), *Retrotransposon-Based Insertion* Polymorphism (RBIP) markers, Single Nucleotide Polymorphism (SNP) marker, and Targeted Induced Local Lesions In Genomes (TILLING). These have been established as useful methods for the genetic diversity assessment (Deulvot *et al.*, 2010; Ahmad *et al*., 2015; Tahir *et al*., 2016). Among the most widely-used markers in crop species are the microsatellite [Simple Sequence Repeats (SSRs)] (Sarikamiş *et al*., 2010). SSR technique amplifies repetitive or motifs region (ranging in length from 1-6 or more base pairs) of DNA. The best benefits of microsatellite analysis include the accuracy, high polymorphism, co-dominance and genome coverage (Tahir, 2010; Lateef, 2015). These types of markers are frequently used for genome mapping, estimation of genetic diversity, gene tagging and marker-assisted selection (Smýkal *et al*., 2008). In the majority of the cultivated crops, very limited genome sequence information is

* Corresponding author. e-mail: nawroz.tahir@univsul.edu.iq.

available especially for pea compared to other legume crops (Deulvot *et al*., 2010). The present study was carried out to test the suitability of applying SSR techniques on pea genotypes, and to asses the efficency of the defined markers set for diversity studies in a collection of *Pisum sativum.*

2. Materials and Methods

2.1. Genotypes Collection

A total of twenty-two pea genotypes were investigated in the present work (Table 1); these genotypes were gathered from the Agriculture Research Station, Ministry of Agriculture in Sulaimani, Kurdistan region, Iraq.

Table 1. Name and origins of pea (*Pisum sativum L*.) genotypes examined in this study.

No.	Code	Genotype Name	Origin
1	G1	ORP-2	Iraq
2	G2	ORP-3	Iraq
3	G3	ORP-8	Iraq
4	G4	ORP-10	Iraq
5	G5	ORP-11	Iraq
6	G6	ORP-12	Iraq
7	G7	ORP-13	Iraq
8	G8	ORP-15	Iraq
9	G9	ORP-58	Iraq
10	G10	ORP-D1	Iraq
11	G11	Ns minima	Australia
12	G12	Green sage	USA
13	G13	Oregon	USA
14	G14	Provence	Italy
15	G15	Javor	Australia
16	G16	Legacy	Turkey
17	G17	Karina	Turkey
18	G18	Topaz	Turkey
19	G19	Vada nunheins	Turkey
20	G20	Bolero	Turkey
21	G21	Lancet	Germany
22	G22	Rainer	Turkey

2.2. DNA Extraction

By performing cetyltrimethylammonium bromide (CTAB), Genomic DNA was isolated from fresh pea leaves (Doyle, 1991). Then, the concentration and quality of the extracted DNA were determined by 1% (w/v) agarose gels using a Bio-Rad gel imaging system. Finally, the extracted genomic DNA was diluted to 40 ng/µL and stored at (-20 °C).

2.3. Molecular Marker (SSR) Assay

Out of the twenty-four markers, nineteen primers (Reis and Diogo, 2012; Bouhadida *et al*., 2013; Ahmad *et al.*, 2015) were found to be polymorphic, and those were utilized for diversity and structure studies (Table 2). PCR reaction was conducted in a 20 µL reaction containing 1X PCR buffer, 200 mM dNTPs, 0.40 µL of primer, 4mM $MgCl_2$, 1µL Taq polymerase and 80ng templates DNA. PCR amplification was conducted using Applied Biosystems Thermocycler following the PCR protocol: Initial denaturation at 94ºC for seven minutes, thirty-seven cycles at 94ºC for one minute, 50, 54, 55 and 60ºC for one minute, 72ºC for two minutes and a final extension step at 72ºC for seven minutes. Amplified products were resolved on 2.4% agarose gels at 87 V in 1X TBE buffer, and fragment sizes were determined by 100-bp DNA ladder (Invitrogen, USA).

2.4. Statistical Data Analysis

The scorable bands were coded manually as either present (1) and absent (0). Scored data were applied for the calculation of Jaccard's similarity coefficient using XLSTAT 2017 software (XLSTAT, 2017). The Jaccard's coefficient was converted to dissimilarity. The Jaccard's coefficient was converted to dissimilarity. The dissimilarity matrix was used for unweighted pair-group method with arithmetic averages (UPGMA) dendrogram constructed by using XLSTAT 2017 software. The Polymorphism information content (PIC) for SSR markers was calculated using the following formula: $PIC_i = 1-\Sigma Pij^2$, where PICi is the PIC of marker i; Pij is the frequency of the j^{th} pattern for marker i (Kumari *et al.*, 2013). Gene diversity and major of allele frequency were computed by using Power Marker version 3.25 software (Liu and Muse, 2005). To determine the relationship among different genotypes, the principal component analysis (PCA) was conducted by XLSTAT 2017 software. GenAlEx (version 6.5) software also used to estimate the molecular variance among and within the populations (Peakall and Smouse, 2012). Marker index (MI) and resolving power (RP) are measured according to (Powell *et al*., 1996; Prevost and Wilkinson, 1999). For the population structure, a model analysis was fulfilled to infer the genetic structure and to clarify the number of sub-populations using the software STRUCTURE (version 2.3.4) (Pritchard *et al*., 2000). The number of supposed populations (K) was set from one to ten, and the analysis was repeated two times. For each run, the burn-in and MCMC were fixed to 50,000 each, and iterations were deposited to 5. The run with the maximum likelihood was employed to set genotypes into sub-populations.

Table 2. Name, sequence, and annealing temperature of SSRs primers used in this research.

No	SSR markers	F 5'- 3'	R 5'- 3'	AT
1	A5	GTAAAGCATAAGGGGTTCTCAT	CAGCTTTTAACTCATCTGACA	50
2	A6	CTTAAGAGAGATTAAATGGACAA	CCAACTCATAATAAAGATTCAAA	50
3	AA205	TACGCAATCATAGAGTTTGGAA	AATCAAGTCAATGAAACAAGCA	50
4	AA355	AGAAAAATTCTAGCATGATACTG	GGAAATATAACCTCAATAACACA	50
5	AB53	CGTCGTTGTTGCCGGTAG	AAACACGTCATCTCGACCTGC	50
6	AD61	CTCATTCAATGATGATAATCCTA	ATGAGGTACTTGTGTGAGATAAA	50
7	AA92	AAGGTCTGAAGCTGAACCTGAAGG	GCAGCCCACAGAAGTGCTTCAA	50
8	AD73	CAGCTGGATTCAATCATTGGTG	ATGAGTAATCCGACGATGCCTT	50
9	AA372.1	GAGTGACCAAAGTTTTGTGAA	CCTTGAACCCATTTTTAAGAGT	50
10	D23	ATGGTTGTCCCAGGATAGATAA	GAAAACATTGGAGAGTGGAGTA	50
11	AA5	TGCCAATCCTGAGGTATTAACACC	CATTTTTGCAGTTGCAATTTCGT	50
12	AD59	TTGGAGAATGTCTTCTCTTTAG	GTATATTTTCACTCAGAGGCAC	50
13	A9	GTGCAGAAGCATTTGTTCAGAT	CCCACATATATTTGGTTGGTCA	50
14	X13.1a	GAACTAGAGCTGATAGCATGT	GCATGCAAAAGAACGAAACAGG	50
15	PSGAPA1a	GACATTGCCAATAACTGG	GGTTCTGTTCTCAATACAAG	54
16	PEACP LHPPSa	GTGGCTGATCCTGTCAACAA	CAACAACCAAGAGCAAAGAAAA	54
17	PSBLOX13.2a	CTGCTATGCTATGTTTCACATC	CTTTGCTTGCAACTTAGTAACAG	54
18	PSCAB66a	CACACGATAAGAGCATCTGC	GCTTGAGTTGCTTGCCAGCC	54
19	PSMPSAA278b	CCAAGAAAGGCTTATCAACAGG	TGCTTGTGTCAAGTGATCAGTG	60

3. Results and Discussion

3.1. Diversity Parameters

The SSRs are suitable markers of choice employed by different researchers for genetic variation analysis in different crops (Sarikamiş *et al*., 2010). In the present study, twenty-four SSRs primers were employed for the studying of twenty-two pea genotypes. Nineteen SSRs primers exhibited clear fragments and polymorphism on profiling (Tables 3). Nineteen SSRs makers produced sixty-eight polymorphic alleles with an average of 3.789, ranging from two to eight per primer. The level of polymorphism reported here is consistent with the data obtained in Burstin *et al.*(2001) where 3.6 alleles per polymorphic marker were observed for thirty-one markers derived from gene sequences, even though the panel included twelve genotypes. Cupic *et al.* (2009) reported an average of 4.5 alleles per locus using thirty SSRs markers in a population of eighteen pea accessions. However, a higher mean of 5.9 alleles per locus was detected in twenty pea varieties and fifty-seven wild pea accessions using ten SSRs markers (Nasiri *et al.,* 2010). This greater mean of alleles per locus may be due to the wild pea genotypes in their study thus making the average of total detected alleles higher. In this study, the highest number of polymorphic bands were stated for the locus AA5 (8 alleles), and the minimum was detected for A6, AD73, AA205 and PSMPSAA278b markers (2 alleles). Similarly, Nasiri *et al.* (2010) observed 8 alleles for marker AF004843. The number of unique positive bands was 7 across all genotypes. The maximum number of unique bands was recorded by PEACPLHPPSa (2 bands). PIC, marker index and resolving power values indicate the relative informativeness of each marker and the average PIC, marker index and resolving power values, in the present research, were found to be 0.513, 2.090 and 2.703, respectively. PIC, marker index and resolving power values ranged between 0.228 for PSCAB66a to 0.935 for AA5, 0.507 for A6 to 7.476 for AA5 and 0.636 for AA205 to 7.636 for AA5, respectively. This result suggests that alleles of marker AA5 were uniformly distributed among pea genotypes. In the present study, the high value of polymorphism parameters is owing to the efficiency of the selected SSRs primers. Heterozygosity was detected to be very low which may be due to autogamous nature of pea. Gene diversity calculated according to Nei (1973) varied from 0.298 (A6) to 0.938 (AA5) with the average of 0.562 (Table 3). Shannon index was determined, and was stated between 0.276 and 2.416 with a mean of 0.833. Loridon *et al.* (2005) obtained a PIC value of 0.73 for locus AA206. The PIC in this research is higher than that reported by Burstin *et al.* (2001) (four alleles). Ahmad *et al*. (2015) stated that marker AA121 was highly informative and had the maximum level of polymorphism with the highest PIC value of 0.887 and a resolving power value of 0.901.

Table 3. Details of 19 SSRs primers and their genetic diversity parameters used for genotyping in the 22 pea genotypes.

Primers	TAF	TPB	TPB%	Size	NUB	Major Allele Frequency	Gene Diversity	PIC	Marker index	Resolving power	H
A5	4	4	100.000	200-1000	0	0.591	0.603	0.569	2.276	3.909	0.931
A6	2	2	100.000	150-180	0	0.818	0.298	0.253	0.507	2.000	0.474
A9	6	5	83.333	340-1200	0	0.455	0.736	0.709	3.546	2.727	1.264
AA205	3	2	66.667	260-660	0	0.682	0.479	0.427	0.854	0.636	0.276
AA355	4	3	75.000	190-720	1	0.636	0.541	0.496	1.489	2.818	0.528
AA372.1	5	4	80.000	225-1150	0	0.318	0.777	0.744	2.974	3.273	1.037
AA430902a	2	2	100.000	420-450	0	0.545	0.496	0.373	0.746	2.000	0.689
AA5	8	8	100.000	180-840	0	0.091	0.938	0.935	7.476	7.636	2.416
AA92	8	5	62.500	150-950	1	0.364	0.744	0.706	3.530	2.636	1.119
AB53	5	4	80.000	250-1200	1	0.591	0.545	0.471	1.884	2.818	0.517
AD59	5	4	80.000	530-1240	0	0.818	0.322	0.311	1.245	2.273	0.316
AD61	7	7	100.000	120-500	1	0.364	0.806	0.786	5.505	6.364	1.493
AD73	3	2	66.667	220-275	0	0.545	0.591	0.522	1.045	1.909	0.653
D23	3	3	100.000	150-200	0	0.409	0.649	0.574	1.721	2.000	1.070
PEACP LHPPSa	5	3	60.000	150-650	2	0.818	0.318	0.302	0.905	2.000	0.368
PSCAB66a	4	3	75.000	200-700	1	0.864	0.244	0.228	0.685	0.455	0.576
PSGAPA1a	4	3	75.000	150-1200	0	0.409	0.702	0.653	1.958	1.909	0.842
PSMPSAA278b	2	2	100.000	180-200	0	0.727	0.397	0.318	0.636	2.000	0.586
X13.1a	3	2	66.667	250-750	0	0.591	0.483	0.367	0.733	2.000	0.677
Total	83	68	81.928		7						
Mean	4.368	3.789				0.560	0.562	0.513	2.090	2.703	0.833

TAF: Total number of fragments, TPB: Total number of polymorphic bands, NUB: Number of unique bands, PIC: Polymorphism information content, H: Shannon Index.

3.2. Clustering and Genetic Relationship

Clustering and principal component analysis (PCA) avail as a platform to supply a spatial clarification of the comparative genetic dissimilarity between the genotypes. It also estimates the robustness of the differentiation among the clusters assorted by the dendrogram (Liu *et al.*, 2013). A fan dendrogram of the SSRs data demonstrated clear groupings of pea genotypes on the basis of origin (Figure 1). The UPGMA depended on cluster analysis for SSRs alleles which exhibited that twenty-two genotypes were clustered into three major clades at the dissimilarity coefficient of 0.520. Clade I had four genotypes: G3 (ORP-8), G8 (ORP-15), G7 (ORP-13) and G11 (Ns minima). Clade II is the large cluster including thirteen genotypes which are represented by five sub-groups at a dissimilarity coefficient of 0.45. The first sub-group consisted of G17 (Karina), G18 (Topaz), G19 (Vada nunheins), G20 (Bolero) and G22 (Rainer). G10 (ORP-D1), G4 (ORP-10), G14 (Provence) and G13 (Oregon) belonging to sub-group two. The third sub-group composed of G12 (Green sage) and G15 (Javor) while the two genotypes G16 (Legacy) and G21 (Lancet) created the fourth and fifth sub-groups, respectively. Group III could be divided into two sub-clades at a dissimilarity coefficient of 0.48. The subgroup I consisted of two genotypes, *viz.*, G2 (ORP-3) and G9 (ORP-58). The subgroup II comprised G1 (ORP-2), G5 (ORP-11), and G6 (ORP-12). The minimum genetic dissimilarity of 0.179 was found between G12 (Green sage) and G15 (Javor) followed by 0.216 between G18 (Topaz) and G19 (Vada nunheins). The maximum genetic dissimilarity of 0.707 was found between G5 (ORP-11) and G12 (Green sage) followed by 0.705 between G5 (ORP-11) and G21 (Lancet). Ahmad *et al.* (2015) scored a range of 0.075-0.875 of genetic similarity among thirty-five accessions of pea using SSRs markers.

In our PCA scatter plots, the first two principal components stated 9.80 and 16.70% of the total variation, respectively. In accordance with the dendrogram result, pea genotypes were clearly separated into three major clusters (Figure 2). As shown in Figures 1 and 2, the genotype G1 (ORP-2), G5 (ORP-11) and G6 (ORP-12), and G7 (ORP-13) were far distant genotypes from the others. These results reveal that the genotypes taken in the study are genetically diverse. The genetic distance identified in this research can be employed in the crossing programs. The number of clusters in the current study is higher than that obtained by Bouhadida *et al.* (2013) (2 clusters) and Reis and Diogo (2012) (2 clusters), and is lower than that stated by Ahmad *et al.* (2015) (4 clusters).

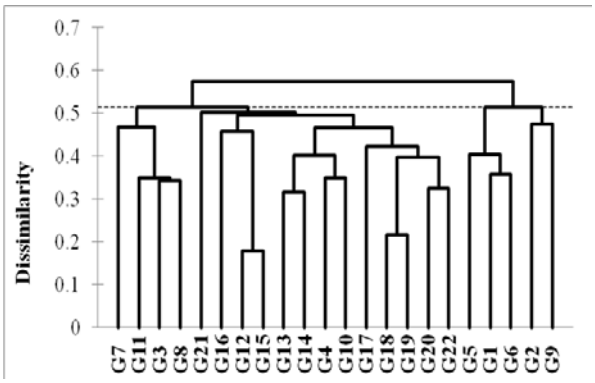


Figure 1. Clustering of 22 genotypes using UPGMA based on SSRs data. Genotypes: G1:ORP-2, G2:ORP-3, G3:ORP-8, G4:ORP-10, G5:ORP-11, G6:ORP-12, G7:ORP-13, G8:ORP-15, G9:ORP-58, G10:ORP-D1, G11:Ns minima, G12:Green sage, G13:Oregon, G14:Provence, G15:Javor, G16:Legacy, G17:Karina, G18:Topaz, G19:Vada nunheins, G20:Bolero, G21:Lancet and G22:Rainer.

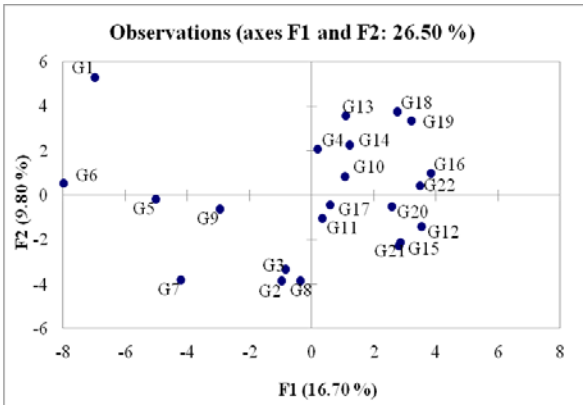


Figure 2. PCA of 22 pea genotypes based on 19 SSRs loci. The two PCA axes accounted for 16.70 and 9.80 % of the total genetic variation. Genotypes: G1: ORP-2, G2: ORP-3, G3: ORP-8, G4: ORP-10, G5: ORP-11, G6: ORP-12, G7: ORP-13, G8: ORP-15, G9: ORP-58, G10: ORP-D1, G11: Ns minima, G12: Green sage, G13: Oregon, G14: Provence, G15: Javor, G16: Legacy, G17: Karina, G18: Topaz, G19: Vada nunheins, G20: Bolero, G21: Lancet and G22: Rainer.

3.3. Analysis of Genetic Variation Among Pea Genotypes using AMOVA

For the assessment of genetic differentiation among the tested pea genotypes six populations (in term of origins), analysis of molecular variance (AMOVA) was conducted. The result exhibited that the pea genotypes were significantly distinct from their relatives at P-value of 0.002 (Table 4). The variance among the populations clarified 14% and within the populations illustrated 86% of genetic divergence. The pair-wise PhiPT value (which is corresponding to FST in the assessment of genetic differentiation) was 0.139 and denoted relatively large deal of discrimination among pea populations. This result indicated that the degree of variation within the population is higher than that obtained among populations suggesting the existence of a significant number of specific and rare alleles and a divergence in allele frequencies among the genotypes. In the current research, the variation among populations is lesser than the mean of differentiation among populations (41%) reported by Teshome *et al*. (2015) in the Ethiopian field pea. Wang *et al*. (2015) has demonstrated 41% of differentiation among populations in 266 grass pea accessions. The differentiation within the population in this study is far greater than that found by (Teshome *et al.*, 2015; Wang *et al*., 2015)

Table 4. AMOVA among six populations (origins) and within population based on 22 SSRs loci of pea genotypes.

Source	df	SS	MS	Est. Var.	%	P
Among Pops	5	78.717	15.743	1.622**	14%	0.002
Within Pops	18	180.200	10.011	10.011**	86%	0.002
Total	23	258.917		11.633	100%	

3.4. Population Structure Analysis

Based on nineteen SSRs markers, the estimation of the population structure of twenty-two pea genotypes was performed by STRUCTURE software with Pritchard correction. The structure simulation with STRUCTURE HARVESTER displayed that the L (*K)* value had the maximum peak at $K = 2$ (Figure 3), inferring that two populations can incorporate all individuals from the twenty-two genotypes with the highest likelihood. This suggested the existing of two major model populations, which were visualized in the graph (Figure 4). Based on the membership fractions, the genotypes with the probability of ≥ 80% were assigned to corresponding subgroups or subpopulations with others categorized as an admixture (Table 5). Subgroup-1 or subpopulation-1 included eight genotypes with most of the genotypes being of local origin, and subgroup-2 consisted of eleven genotypes composed of non-Iraqi genotypes. Only three genotypes: G4 (ORP-10), G11 (Ns minima), and G17 (Karina) were considered to be admixture (Table 5). The population differentiation (FST) metric for the subgroup-1 and subgroup-2 was 0.0478 and 0.267 (Table 6), respectively suggesting that the diversity in subgroup-2 genotypes is greater than the variation in subgroup-1. The heterozygosity presenting among genotypes in subpopulation-1 is higher than that existing in subpopulation-2 (Table 6). These results revealed high genetic variation. Teshome *et al.* (2015) have successfully detected nine subgroups in their study of pea population with large admixture genotypes. Wang *et al*., (2015) have identified three subpopulations among 256 pea genotypes. Based on thirty-one SSRs markers, Jain *et al*. (2014) have divided a collection of ninety-six cultivars of pea into four groups.

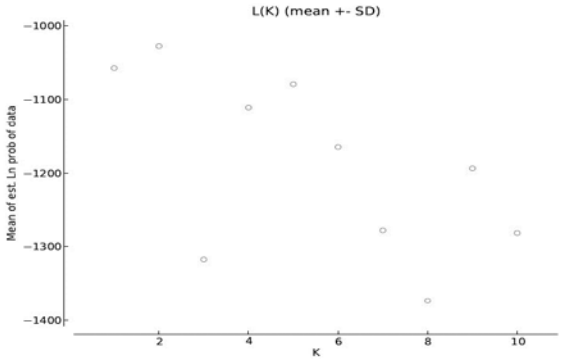


Figure 3. Graph of estimated membership fraction for the optimal value of K for 22 genotypes. The maximum of Ln prob determined by structure harvester is $K = 2$.

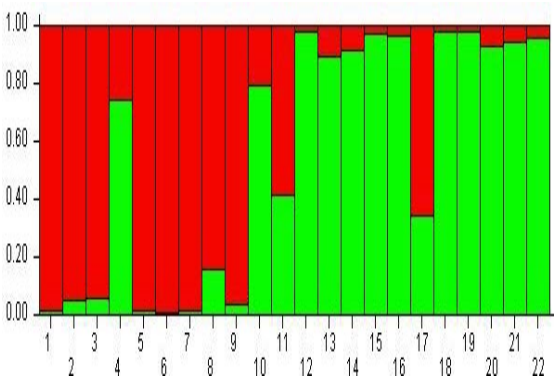


Figure 4. Population structure of 22 pea genotypes organized stand on inferred ancestry based on membership fractions, the genotypes with the probability of ≥ 80 % was set to the corresponding subpopulation, genotypes: 1: ORP-2, 2: ORP-3, 3: ORP-8, 4: ORP-10, 5: ORP-11, 6: ORP-12, 7: ORP-13, 8: ORP-15, 9: ORP-58, 10: ORP-D1, 11: Ns minima, 12: Green sage, 13: Oregon, 14: Provence, 15: Javor, 16: Legacy, 17: Karina, 18: Topaz, 19: Vada nunheins, 20: Bolero, 21: Lancet and 22: Rainer.

Table 5. Population structure cluster of pea genotypes based on inferred ancestry values.

Genotypes	Sub-polpulation-1	Sub-polpulation-2	Inferred cluster
G1	0.984	0.016	1
G2	0.945	0.055	1
G3	0.936	0.064	1
G4	0.254	0.746	1 and 2 (admixture)
G5	0.985	0.015	1
G6	0.986	0.014	1
G7	0.979	0.021	1
G8	0.836	0.164	1
G9	0.961	0.039	1
G10	0.200	0.800	2
G11	0.582	0.418	1 and 2 (admixture)
G12	0.017	0.983	2
G13	0.103	0.897	2
G14	0.084	0.916	2
G15	0.027	0.973	2
G16	0.029	0.971	2
G17	0.655	0.345	1 and 2 (admixture)
G18	0.020	0.980	2
G19	0.016	0.984	2
G20	0.071	0.929	2
G21	0.051	0.949	2
G22	0.036	0.964	2

Genotypes: G1: ORP-2, G2: ORP-3, G3: ORP-8, G4: ORP-10, G5: ORP-11, G6: ORP-12, G7: ORP-13, G8: ORP-15, G9: ORP-58, G10: ORP-D1, G11: Ns minima, G12: Green sage, G13: Oregon, G14: Provence, G15: Javor, G16: Legacy, G17: Karina, G18: Topaz, G19: Vada nunheins, G20: Bolero, G21: Lancet and G22: Rainer.

Table 6. Fixation indices (Fst) and heterozygosity within population created by structure population.

Mean value of Fst-subpopulation-1	0.0478
Mean value of Fst-subpopulation-2	0.267
Heterozygosity of subpopulation-1	0.324
Heterozygosity of subpopulation-2	0.276

4. Conclusions

Based on different statistical analyses including PCA and AHC, the current study identified three clades with 2-3 sub-clusters within twenty-two pea genotypes selected for performing the association mapping panel and breeding program. The allele information and diversity parameters have indicated the existence of a large genetic base in this collection. The output structure analysis in this investigation is not in accordance with the clustering method and principal component analysis. Thus, the results of this study indicate that the determination of genetic variation among pea genotypes by SSR markers can be useful for parental genotype selection in breeding programs.

Acknowledgment

We thank our colleagues from the Kurdistan Institution for Strategic Studies and Scientific Research who provided insight and expertise which greatly assisted with this research.

References

Ahmad S, Kaur S, Lamb-Palmer ND, Lefsrud M and Singh J. 2015. Genetic diversity and population structure of *Pisum sativum* accessions for marker-trait association of lipid content. *Crop J.*, **3**: 238–245.

Bouhadida M, Benjannet R, Madrid E, Amri M and Kharat M. 2013. Efficiency of marker-assisted selection in detection of *ascochyta* blight resistance in Tunisian chickpea breeding lines. *Phytopathol Mediterr.*, **52**: 202–211.

Burstin J, Deniot G, Potier J, Weinachter C, Aubert G and Baranger A. 2001. Microsatellite polymorphism in *Pisum sativum. Plant Breed.*, **120**: 311–317.

Cupic T, Tucak M, Popovic S, Bolaric S, Grljusic S, Kozumplik V and Bieb PM. 2009. Genetic diversity of pea (*Pisum sativum* L .) genotypes assessed by pedigree, morphological and molecular data. *Environment*, **7**: 343–348.

Deulvot C, Charrel H, Marty A, Jacquin F, Donnadieu C, Lejeune-Hénaut I, Burstin J and Aubert G. 2010. Highly-multiplexed SNP genotyping for genetic mapping and germplasm diversity studies in pea. *BMC Genomics*, **11**: 468.

Doyle J. 1991. CTAB Total DNA Isolation. Mol. Tech. Taxon. 283–293.

Hollingsworth M. 2006. **DNA Fingerprinting in Plants: Principles, Methods**, **and Applications,** 2nd Edn. Weising K, Nybom H, Wolff K, Kahl G. 2005. Boca Raton: CRC Press.

Jain S, Kumar A, Mamidi S and McPhee K. 2014. Genetic diversity and population structure among pea (*Pisum sativum* L.) cultivars as revealed by simple sequence repeat and novel genic markers. *Mol Biotechnol.*, **56**: 925–938.

Kumari P, Basal N, Singh AK, Rai VP, Srivastava CP and Singh PK. 2013. Genetic diversity studies in pea (*Pisum sativum* L.) using simple sequence repeat markers. *Genet Mol Res*., **12 (3):** 3540-3550.

Lateef DD. 2015. DNA marker technologies in plants and applications for crop improvements. *J Biosci Med.,* **3**: 7–18.

Liu K and Muse SV. 2005. PowerMarker: an integrated analysis environment for genetic marker analysis. *Bioinformatics*, **21(9)**: 2128–2129.

Liu SR, Li WY, Long D, Hu CG and Zhang JZ. 2013. Development and characterization of genomic and expressed SSRs in citrus by genome-wide analysis M. Sun [ed.],. *PLoS One*, **8**: e75149.

Loridon K, McPhee K, Morin J, Dubreuil P, Pilet-Nayel ML, Aubert G, Rameau C, Baranger A, Coyne C, Lejeune-Hènaut I and Burstin J. 2005. Microsatellite marker polymorphism and mapping in pea (*Pisum sativum* L.). *Theor Appl Genet.,***111**: 1022–1031.

Nasiri J, Haghnazari A and Saba J. 2010. Genetic diversity among varieties and wild species accessions of pea (*Pisum sativum* L.) based on SSR markers. *African J Biotechnol.,* **8**: 3405–3417.

Nei M and Genetics P. 1973. Analysis of gene diversity in subdivided populations. *Proc Nat Acad Sci.,***70**: 3321–3323.

Peakall R and Smouse PE. 2012. GenALEx 6.5: Genetic analysis in Excel. Population genetic software for teaching and research-an update. **28**: 2537–2539.

Powell, W, Morgante M, Andre C, Hanafey M, Vogel J, Tingey S and Rafalski A. 1996. The comparison of RFLP, RAPD, AFLP and SSR (microsatellite) markers for germplasm analysis. *Mol Breed.,* **2**: 225–238.

Prevost A and Wilkinson MJ. 1999. A new system of comparing PCR primers applied to ISSR fingerprinting of potato cultivars. *Theor Appl Genet.***, 98**: 107–112.

Pritchard JK, Stephens M and Donnelly P. 2000. Inference of population structure using multilocus genotype data. *Genetics,* **155**: 945–959.

Reis CMG and Diogo MG. 2012. Identification of field pea cultivarsusing microsatellite molecular markers. *Plant Pysiol Genet.*, **2**: 57–63.

Sarikamiş G, Yanmaz R, Ermiş S, Bakir M and Yükse lC. 2010. Genetic characterization of pea (*Pisum sativum*) germplasm from Turkey using morphological and SSR markers. *GenetMol Res.*, **9**: 591–600.

Smýkal P, AubertG, BurstinJ, Coyne CJ, Ellis NTH, Flavell AJ, Ford R, Hýbl M, Macas J, Neumann P, McPhee KE, Redden RJ, Rubiales D, Weller JL and Warkentin TD. 2012. Pea (*Pisum sativum* L.) in the Genomic Era. *Agronomy*, **2**: 74–115.

Smýkal P, Horáček J, Dostálová R and Hýbl M. 2008. Variety discrimination in pea (*Pisum sativum* L.) by molecular, biochemical and morphological markers. *J Appl Genet.*, **49**: 155–166.

Tahir NA, Hamakareem HF and Hama-Amin BO. 2016. Differentiate of ten pea (*Pisum sativum* L) cultivars by RAPD markers and seeds storage proteins. *JJAS*, **11**: 95–107.

Tahir NA. 2010. Germination characteristics and molecular characterizations of some wheat varieties in Sulaimanyah by SSR marker. *Turkish J Biol.,* **34**: 109-117.

Teshome A, Bryngelsson T, Dagne K and GeletaM. 2015. Assessment of genetic diversity in Ethiopian field pea (*Pisum sativum* L.) accessions with newly developed EST-SSR markers. *BMC Genet.,***16**: 102.

Wang F, Yang T, Burlyaeva M, Li L, Jiang J, Fang L, Redden R and Zong X. 2015. Genetic diversity of grasspea and its relative species revealed by SSR markers. *PLoS One*, **10**: e0118542.

XLSTAT. 2017. **Data Analysis and Statistical Solution for Microsoft Excel. Addinsoft,** Paris, France.

JJBS

Volume 11, Number 2,June 2018
ISSN 1995-6673
Pages 209 - 214

Jordan Journal of Biological Sciences

Bacterial Causative Agents Associated with Subclinical Mastitic in Dromedary She-Camels in Southeastern Algeria

Leyla Hadef[1,2,*], Hebib Aggad[1] and Brahim Hamad[1,2]

[1]Ibn Khaldoun University of Tiaret, Institute of Veterinary Sciences, Laboratory of Hygiene and Animal Pathology, P.O. Box 78 Tiaret, 14000 Tiaret;

[2]University of El-Oued, Faculty of Nature and Life Sciences, Department of Agronomy, P.O. Box 789 El-Oued, 39000 El-Oued, Algeria

Received October 19, 2017; Revised November 19, 2017; Accepted November 23, 2017

Abstract

The present study was designed to identify the different bacterial species causing the subclinical mastitis in she-camels in Algeria, and to determine their evolution during the lactation period. One hundred and fifty three milk samples were collected from seventeen lactating she-camels from southeastern Algeria. The results showed that, 106 (69.28%) of the 153 samples examined contained bacteria, of which 84 (54.90 %) gave pure culture, 21 (13.72%) gave mixed isolations of two types of bacteria or more, and 1 (0.65%) showed contamination of culture. Furthermore, the percentage of the sub-clinical mastitis cases in the Algerian camels was not significantly different ($p > 0.05$) on the basis of the lactation stage. On the other hand, the Bacteriological examinations of the milk samples revealed that coagulase negative staphylococci (CNS) were the most predominant species causing the subclinical mastitis in she camels 39 (46.43%), followed by *Enterobacteriaceae* at 16 (19.05%), coagulase positive staphylococci (CPS) 15 (17.86%), and *Micrococcus* spp. at 6 (7.14%), in addition to other bacteria types. Among the CNS isolated, *Staphylococcus arlettae* (11.91%) and *Staphylococcus muscae* (9.53%) were the most dominant species. From the CPS species isolated, *Staphylococcus aureus* (7.14%) and *Staphylococcus hyicus* (7.14%) were the major species isolated, and from the *Enterobacteriaceae, E.coli* (10.72%) was the main species isolated. Other species, including *Bacillus cereus, Strentococcus* spp., *Aeromonas hydrophila*, *Achromobacter* spp. and *Flavobacterium* spp. were also isolated with low percentages**.**

Keywords: Algeria, Bacteria, She-camels, Subclinical mastitis.

1. Introduction

The dromedary camel (*Camelus dromedarius*) is the most important livestock species in the desert and semi-desert areas of Northern and Eastern Africa as well as in the deserts of the Arabian Peninsula (Al-Juboori *et al*., 2013). On the whole, camels are considered as the main source of both milk and meat production in these areas (Lyer *et al*., 2014).

The she camel milk, similar to that of other dairy animals, contains all the essential nutrients and is regarded a perfect nutritious drink (Tuteja *et al*., 2013). Furthermore, it contains a high proportion of antibacterial substances and higher concentrations of vitamin C in comparison with the cow milk (Barlowska *et al*., 2011). People in arid, semi-arid and desert areas consume raw camel milk as one of the main components of their diet (Siboukeur, 2007). This poses a health risk to humans since the milk is a very nutritious medium; readily supporting the growth of microorganisms originating from environmental contaminants (Kotb *et al*., 2010), or as a result of clinical and subclinical mastitis (Wanjohi, 2014).

Mastitis is a complex disease which affects all dairy animals without discrimination. It causes great economic losses if not detected and treated promptly (Lyer *et al*., 2014). Mastitis can be defined as an inflammation of the parenchymal tissue of the mammary gland. Regardless of cause, it is characterized by a range of physical and chemical changes in the milk in addition to pathological changes in the glandular tissue which include swelling, heat, pain, and edema of mammary gland. The most important changes in the milk include discoloration, presence of clots and presence of a large number of Leukocytes (Radostits *et al*., 2007).

Moreover, camel mastitis has been estimated to affect more than 40 % of the lactating she-camels (Ahmad *et al*., 2011; Regassa *et al*., 2013). It was also known to cause approximately a 70% loss in milk production (Fazlani *et al*., 2011). The sub-clinical mastitis in she camels is considered as the most prevalent type (Ahmad *et al*., 2011; Alamin *et al*., 2013; Husein *et al*., 2013). It refers to the existence of inflammation with an absence of gross

* Corresponding author. e-mail: : leila.hadef@yahoo.fr.

inflammatory changes in the udder, making it difficult to be diagnosed early. It can be detected only through laboratory testing (Abdel Gadir, 2014). Some reports have indicated that the sub-clinical infection in the udder of dromedary causes lower milk production, and changes in the milk properties which impair the processing and preservation of the milk (Saleh and Faye, 2011). On these accounts, the comprehension of its pathogenesis and the early diagnosis are of vital importance in the treatment of mastitis (Khan *et al*., 2013).

Bacterial infections are the primary causes of mastitis in domestic animals (Seifu and Tafesse, 2010). For this reason, many different bacteria have been isolated from mastitic mammary glands of she-camels. The major pathogens of mastitis in she-camels are *Staphylococcus aureus*, *Streptococcus agalactiae, Bacillus cereus*, *Actinomyces pyogenes*, *Escherichia coli*, *Micrococcus* spp. and *Corynebacterium bovis* (Abdel Gadir, 2014), in addition to, *Streptococcus dysgalactiae* (Husein *et al*., 2013; Regassa *et al*., 2013), coagulase negative staphylococci (Abdurahman, 2006; Husein *et al*., 2013; Wanjohi *et al*., 2013), *Pasteurella* spp., and *Pseudomonas aeruginosa* (Al-Juboori *et al*., 2013).

According to a review study, during the last decades, cases of mastitis in dromedary camels have been reported from many of the camel-rearing counties of Africa and Asia, such as Kenya, Somalia, Sudan, Egypt, Saudi Arabia, Iraq and UAE (Abdel Gadir, 2014). There is slight literature on bovine mastitis, and to a lesser extent on ovine and caprine mastitis in Algeria. However, there is no published data about mastitis in she camels. Accordingly, the present study was undertaken to identify the most significant species of bacteria involved in causing subclinical mastitis in she-camels, and to evaluate the evolution of this disease throughout the lactation period.

2. Materials And Methods

2.1. Study Area

The present study was conducted at Bir Naam, South East of Algeria. This region is characterized by an arid climate with an average summer temperature of 42 °C, and low monthly precipitation of 10.72 mm. The rainy period extends from November to January with a 23.8 mm maximum in January, and pastures in this region are considered arid for the rest of the year. The vegetation commonly consists of steppe plants such as *Stipa tenacissima* and *Ampelodesmos tenax*.

2.2. Management of Camels

This study was conducted from November, 2014 to September, 2015. Seventeen multiparous and healthy lactating she-camels kept under grazing and supplementary farming systems were randomly selected. The she-camels were kept grazing in open areas surrounding the farm from the morning times until mid-day then they were taken inside the farm for milking and for supplemental feeding. They were given supplemental barley concentrate and dry hay straw. The animals were provided with water regularly. The calving occurs mostly during the winter season, starting as early as November. The milking was manual. A good number of the she-camels were infested with the ecto-parasite (ticks).

2.3. Sampling Procedure

The milk samples were collected during three different stages of lactation (early, mid and late respectively). Three samples were taken from each animal at each lactation stage. A total of 153 milk samples were collected for this study. Before the collection of the samples, udders of the she-camels were examined visually, and by palpating for the presence of any lesions, and for redness, pain, heat, and swelling. After that, the camels were allowed for udder preparation by washing with water and by the disinfection of the teats with alcohol 70 °C. The raw camel-milk samples were collected in the early morning in sterilized bottles after eliminating the first streams. The milk samples were labeled, stored in an ice box, and were taken immediately within 2-4 hours after collection to the laboratory for the Bacteriological analysis.

2.4. Bacteriological Analysis

All milk samples were randomly selected, and used for Bacteriological analysis for the detection of specific bacteria causing the sub-clinical mastitis. A loopful of each milk sample was streaked on defibrinated sheep (7%) blood agar, nutrient agar, BCP (Bromcresol Purple Lactose) agar, and Chapman agar. Plates were incubated at 37°C for 24- 48 hours. The grown colonies were subjected to the following tests as recommended by the National Mastitis Council (NMC, 1987): the presumptive identification of the isolates based on the colony morphologic features, Gram stain reaction, hemolytic characteristics, catalase test and biochemical classic tests. Based on the Gram stain technique, there was pure Gram-positive cocci, pure Gram-negative, and mixed isolates. Staphylococci and Micrococci were identified based on their growth characteristics on triple sugar iron agar, mannitol salt agar, nitrate reductase test, urease test, coagulase and catalase tests (Forbes *et al*., 2002; Quinn *et al.*, 2011). Isolates that were tentatively identified as streptococci were evaluated according to growth characteristics on sheep blood agar, catalase production and sugar fermentation tests. Gram-negative isolates were sub cultured on BCP agar and were further tested using triple sugar iron agar, testing motility, the IMViC test (Indole, Methylred, Voges–Proskauer, and citrate utilization tests) and the urease test (Forbes *et al.,* 2002; Quinn *et al.,* 2011). Samples with a growth of 5 or more identical colonies (Contreras *et al*., 1997; Pradieé *et al.,* 2012) were considered positive for subclinical mastitis. The growth of two or more morphological types (> 5 CFU per type) was considered as contamination, and the result was excluded from the analysis (Gonzalo *et al*., 2002; Pradieé *et al*., 2012).

2.5. Statistical Analysis

Data were recorded in Microsoft Excel, 2007 spread sheets for statistical analysis. Descriptive statistics were used to summarize the data and calculate the sample statistics and the various proportions. Additionally, the effect of the stage of lactation on the occurrence of subclinical mastitis was analyzed by Cochran Q Test using SPSS (version 16). Probabilities of $p<0.05$ were considered significant.

3. Results

The nature of the milk samples recorded in the present study is given in Table 1. Out of the 106 culture-positive milk samples examined, 84 (54.90%), 21 (13.73%) and 1 (0.65%), were determined having pure, mixed bacteria with two species or more, or contaminated cultures, respectively. It is clear from the present investigation, that pure infection was common as compared to mixed or contaminated infections (Table 1). On the other hand 47 out of 153 examined milk samples (30.72%) were bacteriologically negative cultures.

Table 1. The bacterial nature of milk samples of the she-camels.

Nature of isolates		Number	Percentage %
Culture-positive samples		106	69.28
Nature of samples	Pure samples	84	54.90
	Mixed samples	21	13.73
	Contamination	1	0.65
Culture-negative Samples		47	30.72
Total		153	100

The data available on the relative frequency of different types of organisms encountered in the udder infection (Table 2) revealed that coagulase negative staphylococci (CNS) were the most important organisms involved in the causes of subclinical mastitis in she-camels (46.43%). *Enterobacteriaceae* came next in significance at (19.05%), followed by coagulase positive staphylococci (CPS) at (17.86%). The percentage of *Micrococcus* spp. was (7.14%), and other bacteria types were low (9.52%) (Table 2).

Table 2. The number and percentage of the predominance of different bacteria in subclinical mastitis of she-camels.

Isolates	Number	Percentage %
Coagulase negative staphylococci (CNS)	39	46.43
Enterobacteriaceae	16	19.05
Coagulase positive staphylococci (CPS)	15	17.86
Micrococcus spp.	6	7.14
Other bacteria	8	9.52
Total	84	100

As indicated in Table 3, the percentage of subclinical mastitis in this study was not affected significantly by the lactation stage (p>0.05).

Table 3. The percentage of subclinical mastitis in she-camels based on the stage of lactation.

Lactation stage (months)	0-2	3-5	6-9	Total
Number of samples	51	51	51	153
Number and percentage of positive samples	29 (56.86 %)	24 (47.06%)	31 (60.78%)	84 (54.90%)
Effect of lactation stage (P-value)	NS	NS	NS	

* p< 0.05, ** p< 0.01, NS: no significant p> 0.05.

Table 4 shows the identification and differentiation of bacterial species causing the subclinical mastitis in she-camels in the current study. The most common isolated CNS species were *S. arlettae* (11.91%), *S. muscae* (9.53%), *S. epidermidis* (5.95%), *S. saccharolyticus* (5.95%), *S. cohnii* (4.77%), *S. succinus* (3.57%), S. *saprophyticus* (2.38%), *S. auricularis* (1.19%) and *S. capitis* (1.19%), respectively. Although, from the CPS species, *S. aureus* (7.14%) and *S. hyicus* (7.14%) were the most dominant causes of subclinical mastitis in she-camels followed by *S. intermedius* (3.57%), respectively. However, from *Enterobacteriaceae***,** the *Escherichia coli* (10.72%) was the most dominant species, followed by *Pseudomonas aeruginosa* (2.38%), *Providencia* spp. (2.38%), *Morganella morganii* (1.19%), *Proteus mirabilis* (1.19%) and *Serratia* spp. (1.19%). Nevertheless other bacteria were isolated such us: *Micrococcus* spp. (7.14%), *Bacillus cereus* (3.57%), *Streptococcus* spp. (2.38%), *Aeromonas hydrophila* (1.19%), *Achromobacter* spp. (1.19%) and *Flavobacterium* spp. (1.19%).

Table 4. The percentage of bacterial species in subclinical mastitis of she-camels.

Gram stain result		Bacteria Species	Number	Percentage %
	Coagulase negative staphylococci (CNS)	*S.arlettae*	10	11.91
Gram positive		*S.muscae*	8	9.53
		S.epidermidis	5	5.95
		S.saccharolyticus	5	5.95
		S.cohnii	4	4.77
		S.succinus	3	3.57
		S.saprophyricus	2	2.38
		S.auricularis	1	1.19
		S.capitis	1	1.19
	Coagulase positive staphylococci (CPS)	*S.aureus*	6	7.14
		S.hyicus	6	7.14
		S.intermedius	3	3.57
	Micrococcus spp.		6	7.14
	Streptococcus spp.		2	2.38
Gram negative	*Enterobacteriaceae*	*Escherichia coli*	9	10.72
		Pseudomonas aeruginosa	2	2.38
		Providencia spp.	2	2.38
		Morganella morganii	1	1.19
		Proteus mirabilis	1	1.19
		Serratia spp.	1	1.19
	Other bacteria	*Bacillus cereus*	3	3.57
		Aeromonas hydrophila	1	1.19
		Achromobacter spp.	1	1.19
		Flavobacterium spp.	1	1.19
		Total	84	100

4. Discussion

In this study, samples containing a single bacterial organism were the most common (54.90%), than those containing two bacterial species or more (13.73%). This finding was consistent with the results of Abdurahman (2006) and Regassa *et al*. (2013), who reported that

compared to mixed infections, pure infections were more common in the milk samples of the she-camels infected with subclinical mastitis. On the other hand, Younan *et al*. (2001) reported that infections in the udder of the lactating camels are quite widespread. Generally speaking, bacteria in the milk can occur through colonization of the teat canal or an infected udder (clinical or subclinical mastitis), or as contaminants (Younan, 2004). Moreover, the current study revealed the presence of tick infestation on udders which causes skin and teat lesions. Furthermore, these lesions facilitate bacterial entry and cause permanent tissue damage (Megersa, 2010).

The results of the present study indicated that the proportion of sub-clinical mastitis cases in camels was not significantly different with the variation of the lactation stage. This result was in agreement with Ali *et al*. (2016). However, Ahmad *et al*. (2011); Aljumaah *et al*. (2011); Husein *et al*. (2013) and Regassa *et al*. (2013) stated that the percentage of mastitis in the early stage of lactation was significantly higher. On the other hand, Suheir *et al.* (2005) declared that the last stage of lactation was found to be associated with a high rate of subclinical mastitis. This variation could be attributed to other factors which were important for predisposing mastitis in she-camels such as the hygienic milking process (Ahmad *et al*., 2011), the use of anti-suckling devices to prevent suckling by the camel's calves, tick bites on the udder, deformities of the udder tissue due to the thorny bushes in the pastoral areas, and camel pox (Abdel-Gadir, 2014), in addition, to age (Ahmad *et al*., 2011), breed, parity number (Abdurhmann, 2006; Ahmad *et al*., 2011; Aljumaah *et al*., 2011) and the production system (Ahmad *et al*., 2011).

Among the bacterial isolates, CNS were identified in this study as the predominant organisms causing subclinical mastitis in she-camels. This finding agreed with the reports of Abdurahman (2006) and Husein *et al*. (2013). However, Wanjohi *et al*. (2013) declared that the most isolated bacterium from mastitic camel's milk in Ethiopia was *Klebsiella*/*Enterobacter* followed by CNS, respectively. Other reports stated that the CPS bacteria were the most dominant causes of mastitis in she-camels (Ahmad *et al*., 2011; Alamin *et al*., 2013; Al-Juboori *et al*., 2013; Regassa *et al*., 2013). CNS bacteria are known as the facultative or minor pathogens isolated from subclinical mastitis (Abdel-Gadir et al., 2005). The frequent occurrence of CNS was most probably due to the contamination of the samples through the teat canal or teat skin. Moreover, CNS were mainly present in air, soil and water of camels (Kotb *et al*., 2010) which served as a source of contamination, and were associated with subclinical mastitis in camels' udders.

The current study suggests that the *Enterobacteriaceae* bacteria were the second cause of subclinical mastitis in she-camels. This finding agreed with that reported by Al-Juboori *et al*. (2013) in UAE. In contrast to the results of this study, many authors reported that *Streptococcus spp.* bacteria were the second most common cause of mastitis in camel herds (Abdurahman, 2006; Seifu and Tafesse, 2010; Ahmad *et al*., 2011; Husein *et al*., 2013; Regassa *et al*., 2013). However, Wanjohi *et al*. (2013) reported that the *Enterobacteriaceae* (*Klebsiella*/*Enterobacter* and *E.coli* species) were the most predominant causes of she-camel mastitis in a study conducted in Ethiopia. The contaminated environment of breeding was the main source of *Enterobacteriaceae* (Kotb *et al*., 2010), which mostly predisposed the udders towards bacterial infections.

The percentage of CPS noted in our study was in agreement with the finding of Suheir *et al*. (2005), but it was lower than that reported by Ahmed *et al*. (2011); Alamin *et al*. (2013) and Wanjohi *et al*. (2013) in milk samples collected from Gharissa districts in Ethiopia. However, the obtained percentage of CPS was higher than that (14.3%) recorded by Wanjohi *et al*. (2013) in milk samples from Wajir districts. The frequency of *Staphylococcus* varied according to different studies, but there is practically no recent or even previous publication on the Bacteriological pathogens in mastitis affecting she-camels where staphylococci are not stated.

The present study also identified a low proportion of *Micrococcus* spp. which was in consistent with the findings of other studies (Saleh and Faye, 2011; Al-Juboori *et al*., 2013), even though Regassa *et al*. (2013) found lower percentage. In contrast to our results, Hawari and Hassawi (2008) recorded that *Micrococcus* spp. bacteria were the predominant mastitis-causing organisms in she-camels. Similarly, Suheir *et al*. (2005) stated that these organisms are an important causative of the mastitis among camels. This bacterium was mainly frequent in the camel environment, particularly, in soil, air and water (Kotb *et al*., 2010), which subjected the milk to the microbial contamination existing in the surroundings elevating the contamination. The low frequency of *Micrococcus* spp. observed in this study could be attributed to the existence of adequate hygiene during the milking and in the management system.

The isolated CNS species in positive samples were *S. arlettae, S. muscae, S. epidermidis, S. saccharolyticus, S. cohnii, S. succinus, S. saprophyticus, S. auricularis* and *S. capitis*. However, Alamin *et al*. (2013) recorded that the commonly isolated CNS species in persistent subclinical mastitis in camels were *S. epidermidis, S. simulans, S. haemolyticus, S. kloosii,, S. lentus , S. delphini, S. saprophyticus, S. lugdunensis, S. sacchrolyticus, S. carnosus* and *S. chromogenes*. In this study, *S. arlettae* and *S. muscae* followed by *S. epidermidis* and *S. saccharolyticus* were the most prevalent causative microorganisms involved in she-camel mastitis, Other authors reported that *S. epidermidis* was the most frequent CNS isolated from the camel milk (Abdel Gadir *et al*., 2005; Al-Juboori *et al*., 2013).

Among the isolated CPS, *S. aureus* and *S. hyicus* were the most predominant species followed by *S. intermedius*. This result was in agreement with Woubit *et al*. (2001), who recorded that S. *aureus* and *S. hyicus* were predominant subclinical mastitis-causing organisms in camels in Ethiopia. However, Alamin *et al*. (2013) reported that *S. aureus* was the main organism involved in causing subclinical mastitis in camels and *S. intermiduis* came next in significance, followed by *S. hyicus*. Other reports suggested that *S. aureus* was the most isolated CPS species (Seifu and Tafesse, 2010; Saleh and Faye, 2011; Al-Juboori *et al*., 2013; Husein *et al*., 2013). This is probably related to the fact that *S. aureus* is well adapted to survive in the udder, and usually establishes a mild subclinical infection of a long duration, from which it shed

in milk, facilitating the transmission to healthy animals, mainly during the milking procedure (Radostits *et al*., 2000).

The relative number of *Streptococcus* spp. isolated in this study, was very similar to that reported by Alamin *et al*. (2013). However, it was much lower than previous studies (Seifu and Tafesse, 2010; Saleh and Faye, 2011; Al-Juboori *et al*., 2013, Husein *et al*., 2013). The low proportion of *Streptococcus* spp. could be explained by the possible premedication of the animals with antibiotics, especially that it is known that mastitis caused by *Streptococcus* spp. is susceptible to eradication via the use of antibiotics (Hawari and Hassawi, 2008; Alqurashi *et al*., 2013).

The percentage of *E. coli* recorded in this study is equal to the earlier findings of Ahmad *et al*. (2011) and Saleh and Faye (2011), but it was much lower than that reported by Wanjohi *et al*. (2013). The low rate of *E. coli* isolates might be partially associated with effective udder washing and drying, post milking teat dipping, and keeping washing towels clean. Because *E. coli* is a common intestinal bacterium, the isolation of this bacterium was taken as indicator of fecal contamination that is contamination of one of the milking hygiene conditions. This is significant because the feces may have contained pathogenic organisms (Wanjohi, 2014). But, camel feces are dry, and do not normally contaminate the udder skin (Eberlein, 2007). However, its presence in milk cannot be avoided completely, but can be rather minimized by good management and hygienic milking practices.

The present study also identified a low rate of *Pseudomonas aeruginosa*, which was in agreement with that reported by Al-Juboori *et al*. (2013). Other bacterial species were isolated including *Providencia* spp., *Serratia* spp., *Proteus mirabilis, Morganella morganii* and *Flavobacterium spp.,* but with very low percentage originating possibly from the camel environment. According to Kotb *et al*. (2010) *Providencia*, *Serratia* and *Proteus* resulted from the contamination of the water and the soil. Moreover, Younan (2004) declared that under pastoral production conditions, environmental contamination was likely to play a bigger role in contaminating the raw camel milk than the initial bacterial contamination of the camel milk. On the other hand, the camel milk has a high antimicrobial activity as compared to that of other animal species, and it's able to inhibit Gram-positive and Gram-negative pathogens. But the natural antimicrobial factors can only provide a limited protection against specific pathogens and for a short period of time (Benkerroum *et al*., 2003).

A low proportion of *Bacillus cereus* was recorded through the present study which complied with the results of most investigations stating that the *B. cereus* species was not a pathogen of importance in camel milk (Ahmad *et al*., 2011; Alamin *et al*., 2013). The high percentage of *B. cereus* reported by Wanjohi *et al*. (2013) could be due to poor management and unhygienic milking practices.

On the whole, the findings of the present study were in accordance with the observations of several previous studies, with only minor variations, possibly attributable to different geographical climates, the management system, and individual variations in susceptibility. However, the failure of some pathogen to grow in the current study such as *Corynebacterium* spp., *Klebsiella* spp., and other mastitis pathogens could be explained by the possible premedication of the infected animals with antibiotics.

5. Conclusion

In conclusion, the results of the current study contribute to the overall knowledge about the main species causing subclinical mastitis in camels in Algeria. It is concluded from this work that pure infection was common in camels as compared to mixed infections and that CNS were the dominant mastitis isolated pathogens, followed by *Enterobacteriaceae*. Furthermore, *S. arlettae, E. coli* and *S. muscae* were the main frequent bacterial isolates from the camels infected with subclinical mastitis in the study area. On the other hand, the present study confirmed that the percentage of sub-clinical mastitis cases in Algerian camels was not significantly different with the difference of the lactation stage. Moreover, adequate hygienic conditions in the environment, identification of subclinical infected glands, clinical treatment of the infected she-camels, dry-period therapy, and performing a systematic regular ecto-parasite control of the livestock are all required in order to minimize the occurrence of subclinical mastitis in the study area and. therefore, reduce the adverse effects of mastitis on the production and quality of camel milk.

Acknowledgements

The authors of the present study are grateful to the camels' owner for agreement to participate in this study. We also like to thank the staff of the Laboratory of Hygiene and Animal Pathology**,** Institute of Veterinary Sciences at Ibn Khaldoun University of Tiaret.

References

Abdel Gadir AE. 2014. Mastitis in camel (*Camelus dromedarius*): past and recent research in pastoral production system of both East Africa and Middle East. *J Vet Med Anim Health*, **6**: 208-216.

Abdel Gadir AE, Hildebrandt G, Kleer JN, Molla B, Kyule M and Baumann M. 2005. Prevalence and risk factors of camel (*Camelus dromedarius*) mastitis based on bacteriological examinations in selected regions of Ethiopia. *J Camel Pract Res,* **12**: 33-36.

Abdurahman OASH. 2006. Udder health and milk quality among camels in the Errer valley of eastern Ethiopia. *Livest Res Rural Dev***, 18**.: 1-9.

Ahmad S, Yaqoob M, Bilal MQ, Muhammad G, Yang LG, Khan MK and Tariq M. 2011. Risk factors associated with prevalence and major bacterial causes of mastitis in dromedary camels (*Camelus dromedarius*) under different production systems. *Trop Anim Health Prod*, **44**: 107-112.

Alamin MA, Alqurashi AM, Elsheikh AS and Yasin TE. 2013. Mastitis incidence and bacterial causative agents isolated from lactating she-camel (*Camelus dromedarius*). *IOSR-JAVS*, **2**: 7-10.

Ali F, Hussain R, Qayyum A, Gul ST, Iqbal Z and Hassan MF. 2016. Milk somatic cell counts and some hemato-biochemical changes in sub-clinical mastitic dromedary she-camels (*Camelus dromedarius*). *Pak Vet J*, **36**: 405-408.

Al-Juboori AA, Kamat NK and Sindhu JI. 2013. Prevalence of some mastitis causes in dromedary camels in Abu Dhabi, United Arab Emirates. *Iraqi J Vet Sci*, **27**: 9-14.

Aljumaah RS, Almutairi FF, Ayadi M, Alshaikh MA, Aljumaah AM and Hussein MF. 2011. Factors influencing the prevalence of subclinical mastitis in lactating dromedary camels in Riyadh Region, Saudi Arabia. *Trop Anim Health Prod*, **43**: 1605-1610.

Alqurashi AM, Alamin MA, Elsheikh AS and Yasin TE. 2013. Sensitivity of bacterial isolates from mastitic she-camel (*Camelus dromedarius*) to antibiotics. *J Am Sci*, **9**: 47-52.

Barlowska J, Szwajkowska M, Litwińczuk Z and Król J. 2011. Nutritional value and technological suitability of milk from various animal species used for dairy production. *Comp Rev in Food Sci Food Safety*, **10**: 291-302.

Benkerroum N, Boughdadi A, Bennani N and Hidane K. 2003. Microbiological quality assessement of Morroccan camel's milk and identification of predominating lactic acid bacteria. *World J Microbiol Biotechnol*, **19**: 645-648.

Contreras A, Corrales JC, Sanchez A and Sierra D. 1997. Persistence of subclinical intramammary pathogens in goats throughout lactation. *J Dairy Sci*, **80**: 2815-2819.

Eberlein V. 2007. Hygienic status of camel milk in Dubai (United Arab Emirates) under tow different milking management systems. Doctorate thesis, Munchen University, Germany.

Fazlani SA, Khan SA, Faraz S and Awan MS. 2011. Antimicrobial susceptibility of bacterial species indentified from mastitic milk samples of camel. *Afr J Biotechnol*, **10**: 2959-2964.

Forbes BA, Sahm DF and Weissfield AS. 2002. **Bailey and Scotts Diagnostic Microbiology,** 11th ed. Mosby Inc, Missouri.

Gonzalo C, Ariznabarreta A, Carriedo JA and San Primitivo F. 2002. Mammary pathogens and their relationship to somatic cell count and milk yield losses in dairy ewes. *J Dairy Sci*, **85**: 1460-1467.

Hawari AD and Hassawi F. 2008. Mastitis in one humped she-camels (*Camelus dromedarius*) in Jordan. *J Biol Sci*, **8**: 958- 961.

Husein A, Berihu H, Abbisalem H and Asamenew T. 2013. Prevalence of camel (*Camelus dromedaries*) mastitis in Jijiga Town, Ethiopia. *Afr J Agric Res*, **8**: 3113-3120.

Khan A, Hussain R, Javed MT and Mahmood F. 2013. Molecular analysis of virulent genes (*coa* and *spa*) of *Staphylococcus aureus* involved in natural cases of bovine mastitis. *Pak J Agric Sci*, **50**: 739-743.

Kotb S, Sayed M and Abdel-Rady A. 2010. Sanitary conditions of lactating dromedary she-camel environment with special reference to milk quality and subclinical mastitis monitoring. *Emir J Food Agric*, **22**: 207-215.

Lyer AP, Albaik M and Baghalallb I. 2014. Mastitis in camel in African and Middle East countries. *J Bacteriol Parasitol*, **5**: 188.

Megersa B. 2010. An epidemiological study of major camel diseases in the Borana Lowland, Southern Ethiopia. *Dry Lands Coordination Group Report*, **58**: 32-33.

NMC. 1987. **Laboratory and Field Handbook on Bovine Mastitis**. National Mastitis Council (NMC) Inc, Madison.

Pradieé J, Moraes CR, Gonçalves M, Vilanova MS, Corrêa GF, Lauz OG, Osorio MTM and Schmidt V. 2012. Somatic cell count and California castitis cest as a diagnostic tool for subclinical mastitis in ewes. *Acta Sci Vet*, **40**: 1038.

Quinn PJ, Markey BK, Leonard FC, Hartigan P, Fanning S and FitzPatrick ES. 2011. **Veterinary Microbiology and Microbial Disease**, 2nd ed. John Wiley and Sons Ltd, Chicester, UK.

Radostits OM, Gay CC, Blood DC and Hinchcliff KW. 2000. **Veterinary Medicine**, 9th ed. Harcourt Publisher Ltd, London.

Radostits OM, Gay C, Hinchcliff KW and Constable PD. 2007. **Veterinary Medicine, A Textbook of the Diseases of Cattle, Horses, Sheep, Pigs and Goats**. 10th ed. Elsevier Saunders, Spain.

Regassa A, Golicha G, Tesfaye D, Abunna F and Megersa B. 2013. Prevalence, risk factors, and major bacterial causes of camel mastitis in Borana Zone, Oromia Regional State, Ethiopia. *Trop Anim Health Prod*, **45**: 1589-1595.

Saleh SK and Faye B. 2011. Detection of subclinical mastitis in dromedary camels (*Camelus dromedarius*) using somatic cell counts, California mastitis test and udder pathogen. *Emir J Food Agri*c, **23**: 48-58.

Seifu E andTafesse B. 2010. Prevalence and etiology of mastitis in traditionally managed camels (*Camelus dromedarius*) in selected pastoral areas in eastern Ethiopia. *Ethiop Vet J*, **14**: 103-113.

Siboukeur O. 2007. Study of camel milk locally collected: physicochemical and microbiological characteristics; Abilities for coagulation (in French). Doctorate thesis, National Institute of Agronomy, El-Harrach, Algiers, Algeria.

Suheir IA, Salim MO and Yasin TE. 2005. Bacteria, Mycoplasma and Fungi associated with sub-clinical mastitis in Camel. *Sudan J Vet Res*, **20**: 23-31.

Tuteja FC, Dixit SK, Patil NV, Suchitra Sena D and Singh S. 2013. **Camel Mastitis**. A technical bulletin (Indian Council of Agricultural Research, Krishi Bhawan, N. Delhi) National Research Centre on Camel, Jorbeer, Shivbari.

Wanjohi M, Gitao CG and Bebora L. 2013. Subclinical mastitis affecting hygienic quality of marketed camel milk from North-Eastern Province, Kenya. *Microbiol Res Int*, **1**: 6-15.

Wanjohi M. 2014. Occurrence of subclinical mastitis, brucellosis and factors responsible for camel milk contamination in Garissa and Wajir districts of north-eastern Kenya. MSc. thesis, University of Nairobi. Kenya.

Woubit S, Bayleyegn M, Bonnet S and Baptiste J. 2001. Camel (*Camelus dromedarius*) mastitis in Borena lowlands pastoral area, Southwestern Ethiopia. *Rev Elev Med Vet Pays Trop*, **54**: 207-212.

Younan M, Ali Z, Bornstein S and Muller W. 2001. Application of California mastitis test in intra mammary *Streptococcus agalactiae* and *Staphylococcus aureus* infection of camels (*Camelus dromedaries*) in Kenya. *Prev Vet Med*, **51**: 307-316.

Younan M. 2004. Milk hygiene and udder health. ***In***: **Milk and Meat from the Camel. Handbook on Products and Processing.** Farah Z and Fischer A (Eds.), VDF Hochschulverlag, Zürich,pp.67-76.

JJBS

Volume 11, Number 2,June 2018
ISSN 1995-6673
Pages 215 - 221

Jordan Journal of Biological Sciences

The Influence of Physicochemical Parameters on Phosphate Solubilization and the Biocontrol Traits of *Pseudomonas aeruginosa* FP6 in Phosphate-Deficient Conditions

Sasirekha Bhakthavatchalu[1] and Srividya Shivakumar[2,*]

[1] *Department of Microbiology, Acharya Bangalore B School, Off Magadi Road;* [2]*Department of Microbiology, School of Sciences, Jain University, Bangalore - 560 011, India*

Received October 31, 2017; Revised November 22, 2017; Accepted November 26, 2017

Abstract

Phosphorous deficiency is a major constraint to crop production due to the rapid binding of the applied phosphorous into fixed forms making it not available to the plants. The aim of this study is to isolate phosphate solubilizing bacteria, and assess their effect on the growth of cowpea plant. When tested for its phosphate solubilizing potential, *Pseudomonas aeruginosa* FP6, a known biological control agent against several phytopathogens, showed 52.38 % solubilizing efficacy. *P. aeruginosa* FP6 showed optimum phosphate solubilization with glucose (181.75 µg/ml) and ammonium sulphate (184.75µg/ml). The presence of soluble phosphates, with different concentrations of KH_2PO_4 supplemented in Pikovskaya agar media (PVK), suppressed tri-calcium phosphate (TCP) solubilization activity by FP6. When grown on Tris buffered phosphate medium *P. aeruginosa* FP6 showed reduction in biocontrol traits except hydrogen cyanide (HCN) production. In pot experiments *P. aeruginosa* FP6 inoculation with TCP soil amendment (100 mg/ kg) showed significant increase in biometric parameters suggesting that the application of *P. aeruginosa* FP6 along with the right dose of a phosphate fertilizer could be considered as a sustainable substitute to a higher dose of a phosphorus fertilizer for the cowpea cultivation.

Keywords: Phosphate solubilization, *P. aeruginosa* FP6, Cowpea, TCP

1. Introduction

Phosphorus (P) is one the most essential elements for plant growth after nitrogen. Phosphorus plays a significant role in several physiological and biochemical plant activities like photosynthesis, the metabolic process of energy transfer, signal transduction, macromolecular biosynthesis, and respiration chain reactions (Khan *et al.*, 2010). One of the advantages of feeding the plants with phosphorus is to create deeper and more abundant roots. Phosphorus causes early ripening in plants, decreasing grain moisture, improving crop quality and it is a sensitive nutrient to soil pH (Soleimanzadeh, 2010).

Despite its wide distribution, phosphorus is one of the least available and the least mobile mineral nutrients for plants in the soil (Mahdi *et al.*, 2012). Many soils have a high reserve of total phosphorus accounting for about 0.05% of soil content on average; however, only 0.1% of the total phosphorus is available to plants (Sharma *et al.*, 2011). Therefore, phosphatic chemical fertilizers that contain large amounts of soluble phosphorus have been applied to the agricultural fields to maximize the production (Shen *et al.*, 2011). This soluble phosphorus in phosphatic fertilizers is easily and rapidly precipitated to insoluble forms with cations such as Ca^{2+}, Fe^{3+}, Al^{3+}, Co^{2+} or Zn^{2+}, and is adsorbed to calcium carbonate, aluminium oxide, iron oxide, and aluminium silicate depending on the particular properties of the soil (Mittal *et al.*, 2008). This transformation decreases the efficiency of soluble phosphorus to be taken up by the plants, and decreases the effectiveness of the fertilizer resulting in the application of increasing amounts of phosphatic fertilizers in the agricultural fields. This unmanaged use of phosphatic fertilizers has increased the agricultural costs and also instigated negative environmental impacts (Karpagam and Nagalakshmi, 2014). Recently, phosphate solubilizing microorganisms have attracted the attention of agriculturists as soil inoculums to improve the plant growth and yield (Nisha *et al.*, 2014).

Phosphate solubilizing microbes play fundamental roles in biogeochemical phosphorus cycling in the natural and agricultural ecosystems. Phosphate-solubilizing microbes can transform the insoluble phosphorus into soluble forms HPO_4^{2-} and $H_2PO_4^-$ by acidification, chelation, exchange reactions, and polymeric substances formation (Mahidi *et al.*, 2011). Therefore, the use of phosphate-solubilizing microbes in agricultural practices would not only offset the high cost of manufacturing phosphatic fertilizers, but would also mobilize insoluble phosphorus in the fertilizers and soils to which they are applied (Tarkka *et al.*, 2008). Bacteria belonging to

* Corresponding author. e-mail: sk.srividya@jainuniversity.ac.in.

Mesorhizobium, Rhizobium, Klebsiella, Acinetobacter, Enterobacter, Erwinia, Achrobacter, Micrococcus, Aerobacter and *Bacillus* have been reported as phosphate solubilizers, but strains belong to Pseudomonads are considered as efficient phosphate solubilizers due to their biofertilizing and biocontrol properties (Baudoin *et al.*, 2010). The application of phosphate-solubilizing microbes around the roots of plants in soils, and in fertilizers has been shown to release soluble phosphorus, promote plant growth, and protect plants from pathogen infection (Walpola and Yoon, 2012).

2. Materials and Methods

2.1. Bacterial Strain

The phosphate producing bacterium was isolated from the rhizospheric soil of vegetable crops from in and around Bangalore, India. The potential isolate was identified as *P. aeruginosa* by 16S rDNA sequence analysis. The nucleotide sequence of the 16S rRNA of *P. aeruginosa* FP6 has been deposited in the GenBank database under the accession number JN861778 (Sasirekha *et al.*, 2013).

2.2. Phosphate Solubilisation in Different Media

Quantitative estimation of inorganic phosphate solubilization was done in six different types of liquid media reported in literature. Compositions of different media are given in Table 1 (Halder *et al.*, 1991; Kim *et al.*, 1997; Vassileva *et al.*, 1998; Nautiyal, 1999; Pikovskaya, 1948). The 250 mL flasks containing 50 mL of media inoculated with 500 µL bacterial culture (10^8 CFU/mL) was incubated at 28°C upto 7 days. Uninoculated media under a similar set of conditions was used as the control. An aliquot of 5 mL was collected every day and cells were removed by centrifugation at 7,500 x g for 10 min. The phosphorus content in culture filtrates was estimated by Fiske and subbarow method (Fiske and Subbarow, 1925). Phosphate solubilizing activity was expressed in terms of tricalcium phosphate (TCP) solubilization, which in turn represents µg/mL of available orthophosphate as calibrated from the standard curve of KH_2PO_4 (0-100 µg/mL). The pH variation in different media during the growth of *P. aeruginosa* FP6 isolate was also observed. The rest of the experiment was performed using Pikovskaya medium (PVK) with 0.5 % TCP.

A rock phosphate sample (RP-140) having a P_2O_5 (phosphorous pentoxide) content of about 18.8% was used. Quantitative estimation of the phosphate solubilization activity was carried out in a PVK medium amended with 0.5% (w/v) rock phosphate with the other conditions being the same as for TCP solubilization for duration of seven days.

Table 1. Composition of different media used in this study.

Media Components (g/L)	Medium1 (AYG; Halder *et al.*, 1991)	Medium2 (Kim *et al*., 1997)	Medium3 (Vassilev *et al.*, 1998)	Medium 4 (PVK; Pikovskaya, 1948)	Medium 5 (NBRIY; Nautiyal, 1999)	Medium 6 (NBRIP; Nautiyal, 1999)
Glucose	20	10	100	10	10	10
$(NH_4)_2SO_4$	1	-	-	0.5	0.5	0.1
$MgSO_4.7H_2O$	0.5	0.4	0.2	0.1	0.1	0.25
Yeast Extract	0.2	0.5	-	0.5	-	-
KCl	-	-	-	0.2	0.2	0.2
NaCl	-	1	-	0.2	0.2	-
$FeCl_3$	Trace	-	-	-	-	-
$FeSO_4.7H_2O$				0.002	0.002	-
$MnSO_4.H_2O$	Trace	-	-	0.002	0.002	-
$MgCl_2.6H_2O$	-	-	-	-	-	5.0
$CaCl_2$	-	0.2	-	-	-	-
NH_4NO_3	-	1.5	0.5	-	-	-
$ZnSO_4$	-	-	0.004	-	-	-
$Ca_3(PO_4)_5$	-	-	-	5.0	5.0	5.0
pH	6.8	7	5	7	7	7

2.3. Effect of Carbon and Nitrogen Sources on P Solubilisation

The effect of different carbon sources on the P solubilization was done with the addition of 1% of respective sugars (sucrose, maltose, fructose, xylose, galactose) in place of the glucose in the PVK medium. Similarly for determining the effect of different nitrogen sources, ammonium sulphate $(NH_4)_2SO_4$ in PVK medium was replaced by 0.5% of different nitrogen salts such as casein, urea, potassium nitrate (KNO_3), sodium nitrate ($NaNO_3$), ammonium chloride (NH_4Cl) and sodium nitrite ($NaNO_2$).

2.4. Effect of Soluble Phosphate Source on P Solubilisation

The effect of soluble P on the phosphate solubilization ability of the *P. aeruginosa* FP6 was carried out in a PVK medium amended with KH_2PO_4 (10 - 150 mM) for a duration of seven days. The broth was inoculated with a FP6 isolate and incubated at 28°C.

2.5. Effect of Tris on P Solubilization

Sterile Tris buffer (pH, 8.0) at 0, 20, 30, 40, 60, 60, 70 and 80 mM concentrations (Sambrook *et al.,* 1989) was added to the PVK broth. 100 µL of the overnight broth culture of the isolate was inoculated and incubated for 24 h at 37°C. The effect of P solubilization in buffered condition in the presence of Tris buffer in the media was assessed by noting the change in pH, and by estimating the amount of P solubilized. Control media was without Tris buffer.

2.6. Multiple Biocontrol Traits of P. aeruginosa FP6 under Buffered Condition

The effect of buffering on phosphate solubilization and other biocontrol traits of FP6 was determined in 100M Tris-HCl (pH-8.0) buffered minimal medium containing (g/L) glucose-10; tricalcium phosphate [$Ca_3(PO_4)_2$]-5; ammonium sulphate [$(NH_4)_2SO_4$]- 0.5; sodium chloride [NaCl]- 0.2; magnesium sulphate [$MgSO_4.7H_2O$]- 0.1; potassium chloride [KCl]- 0.2; yeast extract- 0.5; manganese sulphate [$MnSO_4.H_2O$]- 0.002; ferrous sulphate [$FeSO_4.7H_2O$]-0.002. The Culture filtrate was screened for its phosphate solubilization ability along with its antagonistic activity, siderophore production (Schwyn and Neilands, 1987), HCN production (Kremer and Souissi, 2001) and IAA production (Bric *et al.,* 1991). The same was compared with non- buffered condition (PVK media).

2.7. Effect of P. aeruginosa FP6 on Biometric Parameters

2.7.1. Inoculum Preparation for Pot Experiment

FP6 was grown in King's B broth for forty-eight hours under shaking condition (150 rpm) at room temperature (28±2°C). Cell pellet was collected by centrifugation and suspended in distilled water to give a final concentration of 10^8 CFU/ mL. The seeds of cowpea, (C-152), were used throughout the study They were surface-sterilized in 70% ethanol for two minutes and in 2% sodium hypochlorite for five minutes, followed by washing (ten times) in sterile distilled water. Surface sterilized cowpea seeds were soaked in aqueous solution containing *P. aeruginosa* FP6 (10^8 CFU/mL) and were left for one hour to allow the bacteria to bind the seeds. Control seeds were soaked in sterile distilled water.

2.7.2. Pot Experiments

A mixture of soil: sand (1:1) was autoclaved at 121°C for fifteen minutes. The autoclaved soil: sand was supplemented with three different levels of TCP and two different levels of single super-phosphate (SSP) as insoluble (bound) and soluble phosphate sources, in six different combinations (Table 3). A total of twelve different treatments, including six combinations of phosphate sources (TCP and SSP) and two levels of inoculum treatment (control and test) with three replications of each, were used for conducting the pot studies on the plant growth enhancement of cowpea. The seedlings were watered daily to maintain the moisture at approximately 60% water holding capacity of the soil. The values were expressed as their mean. All experiments were carried out in triplicate. The effects of promoting the bacterial-growth treatments were assessed by measuring the biometric parameters.

2.7.3. Control and Test Pots

Control pots were supplemented with three different levels of tricalcium phosphate and two different level of single super-phosphate. Surface sterilized seeds were placed in control pots. Test pots contained surface-sterilized cowpea seeds coated with *P. aeruginosa* FP6.

2.8. Statistical Analysis

Each data presented was the mean of three replicates. All data were subjected to one way analysis of variance and the mean difference was compared with the least significant difference (LSD). Comparison with $p< 0.05$ was considered significantly different. The analysis of variance was performed using SPSS (version 18) statistical package and mean comparison were carried out using Duncan's multiple range test.

3. Results

FP6 isolate showed a distinct zone of clearance around the colony on PVK medium after five days of incubation due to solubilization of inorganic phosphate. Maximum solubilization efficiency and an index of 52.38 % and 2.26 were observed on PVK media. The results of the present study demonstrated *P. aeruginosa* FP6 to be a potent phosphate solubilizer.

The analysis of media formulation for phosphate solubilization by *P. aeruginosa* FP6 showed maximum phosphate solubilization in medium 6 (NBRIP) containing 1% glucose and 0.01% $(NH_4)_2SO_4$ (412.5 µg/mL), with a drop in pH from 7.2 to 4.9 on the third day followed by medium 5 (NBRIY) 330 µg/mL (pH 7.2 to 4.7) and medium 4 (PVK) 270 µg/mL with a decrease in pH from 7.2 to 5 (Figure. 1) while pH being stable in control. Low levels of P solubilization were observed in other phosphate media. FP6 also solubilized rock phosphate (202.5 µg /mL) by seven days of incubation. Results showed negative correlation (r^2 = -0.159, p = 0.39) between decrease in pH and phosphate solubilization in PVK medium.

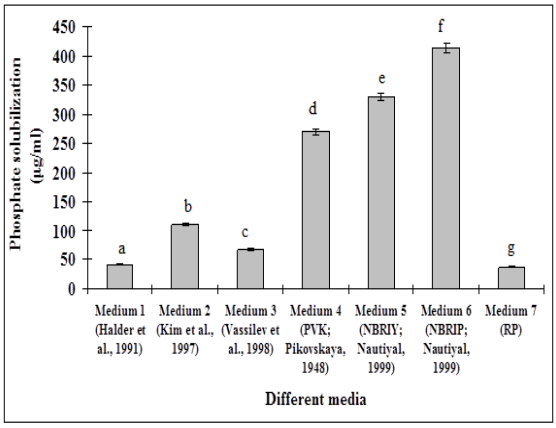


Figure 1. Effect of different media on phosphate solubilization. Different letters above the bars indicate significant difference between different media, according to LSD ($p< 0.05$).

Considering the amount of glucose used in the medium, and the corresponding efficacy of P solubilization, the PVK medium proved to be the most cost effective without compromising the solubilization. Therefore, PVK medium was used for further studies.

3.1. Effect of Carbon and Nitrogen Sources on P Solubilization

The Phosphate solubilization activity of FP6 when evaluated in the presence of five different carbon and seven nitrogen sources by replacing glucose and $(NH_4)_2SO_4$ of the PVK medium showed maximum phosphate solubilization with a decrease in pH in the medium containing glucose (pH 4.85; 181.75 µg/mL) after twenty-four hours of incubation followed by fructose (pH 5.83; 168.25 µg/mL), and xylose (pH 5.64; 57.75 µg/mL) (Figure. 2), whereas sucrose and maltose showed negligible amount of phosphate solubilization with no decrease in pH. A significant negative correlation (r^2 = -0.77, p = 0.04) was observed between phosphate solubilization and the reduction in pH.

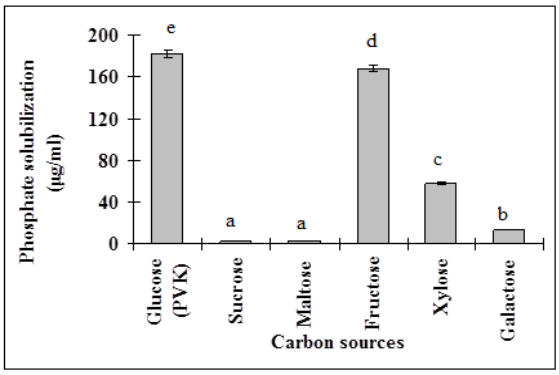


Figure 2. Effect of different carbon sources on phosphate solubilization. Different letters above the bars indicate significant differences between carbon sources, according to LSD ($p< 0.05$).

Nitrogen salts having either ammonium or nitrate groups were used as nitrogen source. Nitrogen salts with ammonium group- ammonium sulphate and ammonium chloride were found to be the best in reducing the medium pH to 4.87 and 5.22, respectively with simultaneous solubilization of 184.75 µg/mL and 169 µg/mL of P (Figure 3) after twenty-four hours of incubation. Other nitrogen sources in the form of urea, nitrate and nitrite failed to support growth of FP6. Though casein showed a pH drop to 4.52, it showed only marginal P solubilization (44.25 µg/mL). Correlation between phosphate solubilization and acidification was found to be statistically significant (r^2 = -0.74, p = 0.038).

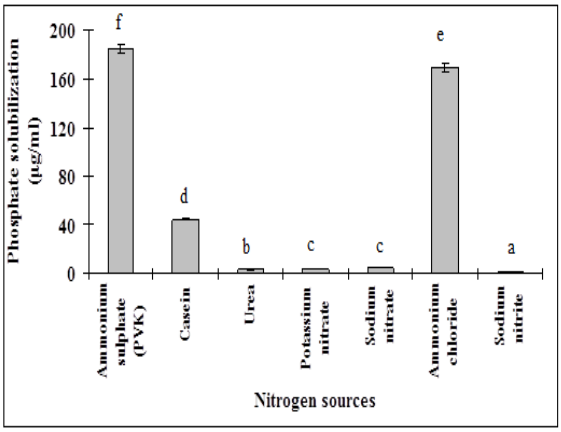


Figure 3. Effect of different nitrogen sources on phosphate solubilization. Different letters above the bars indicate significant differences between nitrogen sources, according to LSD ($p< 0.05$).

3.2. Effect of KH_2PO_4 (free Soluble Phosphate) and Tris on Phosphate Solubilization

The presence of free soluble phosphate in the form of KH_2PO_4 in the medium inhibited phosphate solubilization by FP6 (Figure. 4).The solubilization of phosphate exhibited a negative correlation with a reduction in pH ($r = -0.29$, $r \leq 0.480$).

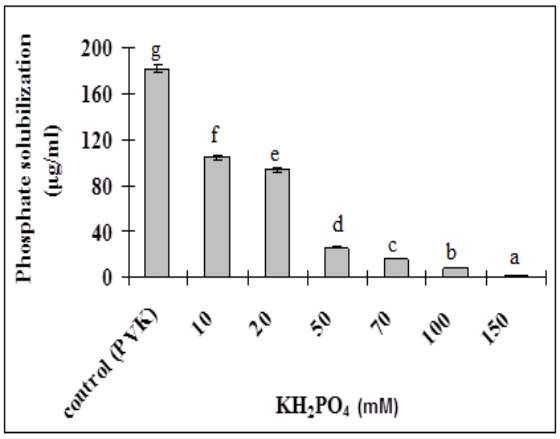


Figure 4. Effect of KH_2PO_4 on phosphate solubilization. Different letters above the bars indicate significant differences between KH_2PO_4 concentrations, according to LSD ($p< 0.05$).

Since the results of the present study showed a drop in pH corresponding to phosphate solubilization, the ability of the strain to solubilize phosphate in different buffering conditions was carried out. Phosphate solubilization was observed up to 80mM Tris, and optimum phosphate solubilization was seen in the presence of 30mM Tris (82.5 µg/mL) with concomitant drop in pH to 4.6. Additions of Tris from 40mM onwards resulted in a drastic decrease in phosphate solubilization with no absolute drop in pH (Figure 5). A statistically significant (r^2 = -0.91, p = 0.0017) negative correlation was developed between the solubilization of phosphate and the decline in pH.

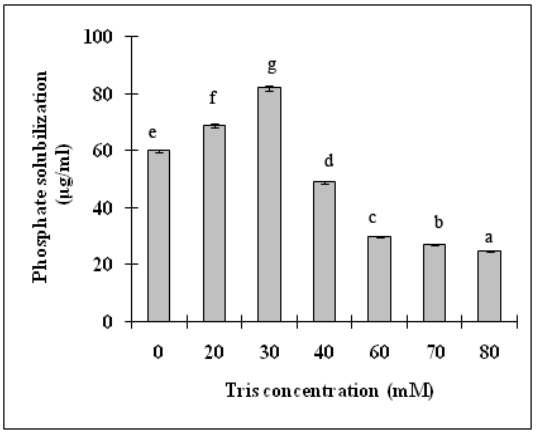


Figure 5. Effect of different concentration of Tris on P solubilization. Different letters above the bars indicate significant differences between Tris concentrations, according to LSD ($p< 0.05$).

3.3. Multiple Biocontrol Traits of P. aeruginosa FP6 under Phosphate Deficient Condition (Buffered Condition)

In the environment, the plant growth is limited by the availability of P despite the abundance of PSBs in the rhizosphere due to the high buffering capacity of soils and the reduced/ loss of P solubilizing efficiency of bacteria under buffered conditions. *P. aeruginosa* FP6 demonstrated P solubilization ability under buffered conditions (upto 80mM Tris). Therefore in the present study we explored biocontrol and PGPR abilities of this strain under phosphate deficient conditions. When grown on Tris-buffered (100mM) PVK media (P deficient), *P. aeruginosa* FP6 showed three fold reductions in IAA, and siderophore production as compared to unbuffered PVK medium (P sufficient). However, a threefold increase in the HCN production was observed in a P-deficient medium (Table 2). There was a concurrence of P-solubilization phenotype, and multiple biocontrol traits in *P. aeruginosa* FP6 under P-deficient conditions, and only the HCN production was found to be independent of the P solubilization trait.

Table 2. Biocontrol traits in PVK medium and Tris buffered PVK medium.

PGPR traits	PVK medium	Tris buffered PVK medium
Phosphate solubilization	310 µg/mL	100 µg/mL
IAA	6 µg/mL	2µg/mL
Siderophore	5 µM	2 µM
HCN (O.D)	0.05	0.15

3.4. Biometric Parameters in the Cowpea Plant

The effect of P on plant growth performance of the cow pea in pot conditions was studied. Compared to the control, a significant increase in all the growth parameters was observed with increasing the concentration of phosphate, but significant difference was not observed, except in shoot weight when treated with 100 and 165 mg/ kg of TCP and superphosphate. Significant increase in shoot length and fresh shoot weight was observed in the combined application of phosphate source (TCP and superphosphate) and *P. aeruginosa* compared to *P. aeruginosa* control indicating the phosphate solubilizing efficacy of the strain (Table 3). However, the combined application of TCP (100 mg/ kg) with *P. aeruginosa* FP6 showed significant a stimulatory effect in biometric parameters compared to control. This increase can be attributed to the absorbance of more P from the soil and its accumulation, resulting in increased shoot length (27.88 cm), root length (10.5 cm), fresh shoot weight (0.51g), and fresh root weight (0.07g). This indicates a positive influence of the combined inoculation regarding the nutrient availability and plant growth.

Table 3. Influence of *P. aeruginosa* FP6 inoculation on the growth of cowpea in the presence of different phosphorus sources

Phosphate treatment (Soil)	Treatment	Shoot length (cm)	Root length (cm)	Fresh weight	
				Shoot weight (g)	Root weight (g)
TCP_0 SSP_0	Control	16.81^{a}	6.69^{a}	0.29^{a}	0.02^{a}
	P. aeruginosa FP6 (test)	21.13^{b}	$8.19^{a,b}$	$0.37^{a,b,c}$	0.02^{a}
TCP_0 SSP_1	Control	20.43^{b}	8^{a}	$0.33^{a,b}$	0.05^{a}
	P. aeruginosa FP6 (test)	$26.31^{c,d,e}$	$9^{a,b}$	$0.42^{b,c,d}$	0.08^{a}
TCP_1 SSP_0	Control	21.13^{b}	$6.59^{a,b}$	$0.33^{a,b}$	0.04^{a}
	P. aeruginosa FP6 (test)	$27.88^{d,e}$	10.5^{b}	$0.51^{d,e}$	0.07^{a}
TCP_1 SSP_1	Control	$23.39^{b,c,d}$	$7.65^{a,b}$	$0.40^{a,b,c,d}$	0.04^{a}
	P. aeruginosa FP6 (test)	$26.88^{d,c}$	$9.54^{a,b}$	$0.49^{c,d}$	0.05^{a}
TCP_2 SSP_0	Control	$24.31^{b,c,d,e}$	$8.6^{a,b}$	$0.37^{a,b,c}$	0.03^{a}
	P. aeruginosa FP6 (test)	$24.4^{b,c,d,e}$	$8.68^{a,b}$	$0.42^{b,c,d}$	0.04^{a}
TCP_2 SSP_1	Control	$22.75^{b,c}$	$7.13^{a,b}$	$0.43^{b,c,d}$	0.02^{a}
	P. aeruginosa FP6 (test)	$25.4^{c,d,e}$	$8.3^{a,b}$	0.55^{e}	0.039^{a}
F value		91.62	115	101.83	6.94
CD @ 0.05 %		3.40	1.17	0.07	0.02

TCP (tri-calcium phosphate), SSP (single superphosphate); Subscripts 0, 1 and 2 for TCP indicate concentrations of 0, 100 and 200 mg/ kg soil respectively. Subscripts 0 and 1 for SSP indicate concentrations of 0 and 165 mg/ kg soil respectively. Values represent mean. Mean values followed by the same letter are not significantly different at the 0.05 level of confidence.

4. Discussion

Phosphorus is one of the most essential elements for the growth of plants, yet it is not a renewable resource, and its future use in agriculture will be impacted by its declining availability and increased cost (Hameeda *et al.*, 2008). P gets precipitated with calcium, iron and

aluminium and becomes unavailable to plants. Theoretical estimates have suggested that the accumulated P in the soil is sufficient to sustain crop yields worldwide for about 100 years (Khan *et al.,* 2010). Phosphatic biofertilizers in the form of microorganisms can help increase the availability of accumulated phosphates for plant growth by solubilization.

Several species of *Pseudomonas* fall within the promising category of growth-promoting rhizobacteria, and a number of them have been studied for their phosphate solubilising activity (Sharma *et al.,* 2011).

The lack of correlation between phosphate solubilization and pH indicated that solubilization of $Ca_3(PO_4)_2$ was not predominantly due to organic acid release, but alternatively, can also be attributed to other mechanisms such as the release of protons accompanying respiration or NH_4^+ assimilation.

Depending on the composition of the bacterial medium and the final pH of the cultured medium, bacterial P solubilization reported in the literature ranges from 31.5 mg/L to 898 mg/L (Ma *et al.,* 2009; Oliveira *et al.,* 2009). The present study is in agreement with the above mentioned reports.

The slow rate of rock phosphate solubilization could be attributed to its structural complexity and particle size, as well as the nature and quantity of the organic acid secreted by the microorganisms, which suggests the good adaptation of this strain to its ecological niche. Furthermore, the inherent ability of a strain to solubilize these natural forms of insoluble rock P can possibly be improved by optimizing the growth parameters.

The results of the present study on the carbon source effect are in agreement with those of Son *et al.* (2006) and Pallavi and Gupta (2013) who found glucose to be the best carbon source for phosphate solubilization in *Pseudomonas* species. The role of carbon sources is important in phosphate solubilization because the nature of the produced acid is affected by the carbon source. Glucose and fructose are the most frequent and abundant sugars detected in plant exudates that might possibly affect the microbial population which solubilize insoluble phosphates. The increased phosphate solubilization with glucose may be attributed to the greater availability of the energy source for the growth of the organism and for the acid production.

The present study results on the effect of nitrogen source correlates with earlier reports (Sharma *et al.,* 2013; Karunai Selvi and Ravindran, 2012). The Phosphate solubilization in ammonium salts, as the nitrogen source, was substantially higher than with other nitrogen sources, which suggested a P solubilization by the production of organic acids through NH_4^+/H^+ exchange mechanisms. Relwani *et al*. (2008) have stated that the nitrate increased the solubilization of several phosphates. The nitrate uptake by the cell stimulates acid secretion to compensate the cellular ionic potential. Such a stimulus was not observed in the present study.

The inhibition of phosphate solubilization in the presence of soluble phosphate indicates that the phosphate solubilization ability is a stress-induced response. Similar results have also been reported by Srividya *et al.* (2009). The buffering capacity of the medium reduced the effectiveness of FP6 strain in releasing P from TCP. This could be attributed to the resistance in pH change by the buffering activity of Tris. A similar observation has been reported by Joseph and Jisha (2009).

Joseph and Jisha (2009) reported a loss of the P solubilization in phosphate solubilizing bacteria due to buffering. In contrast to the above-mentioned studies, FP6 isolate showed a considerable amount of phosphate solubilization, and other PGPR ability in buffered condition indicating that FP6 can perform better in acidic soils.

Walpola and Yoon (2013) linked the highest growth performance and P uptake of the mung bean to co-inoculating PSB strains and adding TCP. Several studies have shown increase in plant growth and P uptake due to the addition of phosphate-solubilising bacteria (Velineni and Brahmaprakash 2011; Hameeda *et al.,* 2008). Malviya *et al.* (2011) showed a significant increase in dry matter and the groundnut yield in phosphate solubilizing fungi inoculated soil compared to control soil.

5. Conclusion

The current study highlights the phosphate solubilising ability of *P. aeruginosa* FP6. The use of this strain as bioinoculant could be a sustainable practice to enhance the soil fertility and plant growth. The successful use of phosphate solubilising microorganisms along with indigenously available cheaper sources such as the low-grade rock phosphate (RP) can be economical.

Acknowledgements

The authors thank the administration of Jain University for providing the necessary facilities to carry out this work.

Reference

Baudoin E, Lerner A, Sajjad Mirza M, El Zemrany H, Prigent-Combaret C, Jurkevich E, Spaepen S, Vanderleyden J, Nazaret S, okon Y and Moenne-Loccoz Y. 2010. Effects of *Azospirillum brasilense* with genetically modified auxin biosynthesis gene *ipdC* on the diversity of the indigenous microbiota of the wheat rhizosphere. *Res Microbiol.,* **161**:219- 226.

Bric JM, Bostock RM and Silverstone SE. 1991. Rapid in situ assay for indolacetic acid production by bacteria immobilized on a nitrocellulose membrane. *Appl Environ Microbiol.,* **57**:535-538.

Fiske CH and Subbarow Y. 1925. A colorimetric determination of phosphorus. *J Biol Chem.,* **66**:375- 400.

Halder AK, Mishra AK and Chakarbarthy PK. 1991. Solubilization of inorganic phosphate by *Bradyrhizobium*. *Indian J Experimental Biol.,* **29**:28- 31.

Hameeda B, Harini G, Rupela OP, Wani SP and Reddy G. 2008. Growth promotion of maize by phosphate-solubilizing bacteria isolated from composts and macrofauna. *Microbiol Res.,* **163**:234-242.

Joseph S and Jisha MS. 2009. Buffering reduces phosphate solubilising ability of selected strains of bacteria. *World J Agricultural Sci.,* **5**: 135- 137.

Karpagam T and Nagalakshmi PK. 2014. Isolation and characterization of phosphate solubilizing microbes from agricultural soil. *Inter J Current Microbiol Applied Sci.,* **3**: 601-614.

Karunai Selvi B and Ravindran AD. 2012. Carbon and nitrogen sources on insoluble inorganic phosphate solubilization by *Bacillus subtilis*. *Inter J Advan Biol Res.*, **2:** 441- 445.

Khan MS, Zaidi A, Ahemad M, Oves M and Wani PA. 2010. Plant growth promotion by phosphate solubilizing fungi – current perspective. *Arch Agronomy Soil Sci.*, **56**:73-98.

Kim KY, Jordan D and Kirshanan HB. 1997. *Rahnella aquatilis*, a bacterium isolated from soyabean rhizophere, can solubilize hydroxyapatite. *FEMS Microbiol Lett.*, **153**:273- 277.

Kremer RJ and Souissi T. 2001. Cyanide production by rhizobacteria and potential for suppression of weed seedling growth. *Current Microbiol.*, **43** (3):182- 186.

Ma Y, Rajkumar M and Freitas H. 2009. Inoculation of plant growth promoting bacterium *Achromobacter xylosoxidans* strain Ax10 for the improvement of copper phytoextraction by *Brassica juncea*. *J Environ Manag.*, **90**: 831- 837.

Mahdi SS, Talat MA, Hussain Dar M, Hamid A and Latief Ahmad L. 2012. Soil phosphorus fixation chemistry and role of phosphate solubilizing bacteria in Enhancing its efficiency for sustainable cropping - A review. *J Pure Appl Microbiol.*, **6:**1- 7.

Mahidi SS, Hassan GI, Hussain A and Faisul-ur-Rasool. 2011. Phosphorus availability issue- Its fixation and role of phosphate solubilizing bacteria in phosphate solubilization-Case study. *Res J Agricultural Sci.*, **2**:174-179.

Malviya J, Kiran Singh K and Vaibhavi Joshi V. 2011. Effect of phosphate solubilizing fungi on growth and nutrient uptake of ground nut (*Arachis hypogaea*) plants. *Advan Biores.*, **2**: 110- 113.

Mittal V, Singh O, Nayyar H, Kaur J and Tewari R. 2008. Stimulatory effect of phosphate solubilizing fungal strains (*Aspergillus awamori* and *Penicillium citrinum*) on the yield of chickpea (*Cicer arietinum* L. cv. GPF2). *Soil Biol Biochem.*, **40** :718-727.

Nautiyal C S. 1999. An efficient microbiological growth medium for screening phosphate solubilizing microorganisms. *FEMS Microbiol Lett.*, **170**: 265-270.

Nisha K, Padma Devi SN, Vasandha S and Sunitha kumari K. 2014. Role of phosphorous solubilizing microorganisms to eradicate p- deficiency in plants: a review. *Inter J Scientific and Res Publications*, **4**:1-5.

Oliveira CA, Alves VMC, Marriel IE, Gomes EA, Scotti MR, Carneiro NP, Guimaraes CT, Schaffert RE and Sa NMH. 2009. Phosphate solubilising microorganisms isolated from rhizosphere of maize cultivated in an oxisol of the Brazilian Cerrado Biome. *Soil Biol Biochem.*, **41**:1782- 1787.

Pallavi KP and Gupta PC. 2013. Effect of different carbon and nitrogen sources on solubilization of insoluble inorganic phosphate by psychrotolerant bacterial strains. *The Bioscan* **8**: 1299- 1302.

Pikovskaya RI. 1948. Mobilization of phosphorus in soil in connection with the vital activity of some microbial species. *Mikrobiologiya* **17**:362- 370.

Relwani L, Krishna P and Sudhakara Reddy M. 2008. Effect of carbon and nitrogen sources on phosphate solubilization by a wild-type strain and UV-induced mutants of *Aspergillus tubingensis*. *Current Microbiol.*, **57**: 401- 406.

Sambrook J, Fritsch EF and Maniatis T.1989. **Molecular Cloning: A Laboratory Manual.** 2nd ed. Plainview, New York, Cold Spring Harbor Laboratory Press.

Sasirekha B, Srividya S and Sullia SB. 2013. Characterization of multiple plant growth promotion traits of *Pseudomonas aeruginosa* FP6, a potential stress tolerant biocontrol agents. *Annals of Biol Res.*, **4**:214- 223.

Schwyn B and Neilands JB. 1987. Universal chemical assay for the detection and determination of siderophores. *Analytical Biochem.*, **160**:47- 56.

Sharma SB, Sayyed RZ, Trivedi MH, and Gobi. 2013. Phosphate solubilizing microbes: sustainable approach for managing phosphorus deficiency in agricultural soils. *SpringerPlus*, **2**:587- 600.

Sharma S, Vijay Kumar and Tripathi RB. 2011. Isolation of phosphate solubilizing microorganism (PSMs) from soil. *J Microbiol Biotechnol Res.*, **1**: 90- 95.

Shen J, Lixing Yuan, Junling Zhang, Haigang Li, Zhaohai Bai, Xinping Chen, Zhang W and Zhang F. 2011. Phosphorus dynamics: from soil to plant. *Plant Physiol.*, **156**: 997- 1005.

Soleimanzadeh H. 2010. Effect of VA-Mycorrhiza on growth and yield of sunflower (*Helianthus annuus* L.) at different phosphorus levels. *World Academy of Sci., Eng Technol.*, **4**:11-26.

Son H, Park G, Cha M and Heo M. 2006. Solubilization of insoluble inorganic phosphates by a novel salt- and pH-tolerant *Pantoea agglomerans* R-42 isolated from soybean rhizosphere. *Bioresource Technol.*, **97**: 204- 210.

Srividya S, Soumya S and Pooja K. 2009. Influence of environmental factors and salinity on phosphate solubilization by a newly isolated *Aspergillus niger* F7 from agricultural soil. *Afri J Biotechnol.*, **8**: 1864- 1870.

Tarkka M, Schrey S and Hampp R. 2008. Plant associated micro-organisms. In: **Molecular Mechanisms of Plant and Microbe Coexistence**, Nautiyal CS and Dion P (Eds). Springer, New York. pp 3- 52

Vassileva M, Vassilev N and Azcon R. 1998. Rock phosphate solubilization by *Aspergillus niger* on olive cake-based medium and its further application in a soil plant system. *World J Microbiol Biotechnol.*, **14**: 281- 284.

Velineni S and Brahmaprakash GP. 2011. Survival and phosphate solubilizing ability of *Bacillus megaterium* in liquid inoculants under high temperature and desiccation stress. *J Agricultural Sci Technol.*, **13**: 795- 802.

Walpola BC and Yoon MH. 2012. Prospectus of phosphate solubilizing microorganisms and phosphorus availability in agricultural soils: A review. *Afri J Microbiol Res.*, **6** :6600-6605.

Walpola BC and Yoon MH. 2013. Phosphate solubilising bacteria: Assessment of their effect on growth promotion and phosphorous uptake of mung bean (*Vigna radiata* [L.] R. Wilczek). *Chilean J Agricultural Res.*, **73**: 275-281.

JJBS

Volume 11, Number 2,June 2018
ISSN 1995-6673
Pages 223 - 229

Jordan Journal of Biological Sciences

Insecticidal Toxicity of Goat Weed, *Ageratum conyzoides,* Linn. (Asteraceae) against Weevil, *Dermestes maculatus,* Degeer (Coleoptera: Dermestidae) Infesting Smoked Fish

Ito E. Edwin[1,*] and Utebor E. Kester [2]

[1]*Tropical Disease Research Unit, Department of Animal and Environmental Biology, Delta State University, Abraka;*

[2]*Department of Biology, College of Education Agbor, Delta State, Nigeria.*

Received August 7, 2017; Revised November 21, 2017; Accepted November 27, 2017

Abstract

In Nigeria and most West African countries, the most common pest of animal products which also infest smoked-dry fish is *Dermestes maculatus* (fish weevil). The bio-insecticidal potentials of *Ageratum conyzoides* (goat weed) leaf powder and extract on fish weevils was evaluated with different doses of the plant materials (0.5 – 3.0 g and 50.0-100 mg^{-ml}). Twenty unsexed adult weevils were exposed to these plant treatments which were all replicated thrice. The mortality rate was observed daily for 120 hours and 72 hours (h) with the use of the powder and extract respectively. The results of this study showed that mortality increased with the increase in gram (g) and the extract (mg^{-ml}) concentration of *A. conyzoides*. Statistically, this indicates that the application of the *A. conyzoides* powder of different concentrations had a significant effect ($P < 0.05$; F = 13.69) on the mean percentage mortality of *D. maculatus* over a 120-hour period of exposure with a Median Lethal Concentration (LC_{50}) of 0.59 g, and Median Lethal Time (LT_{50}) of 22.80 h at 3.0 g. Comparatively, the extract application had no significant effect ($P > 0.05$) on the mean percentage mortality of *D. maculatus* over a seventy-two-hour exposure ($P = 0.2573$; F = 1.7422). The minimum LC_{50} of the extract required to kill 50% of *D. maculatus* was determined as 36.86 mg^{-ml}. The overall results showed that the extract was more toxic than the powder. The effectiveness of the phytochemical components of *A. conyzoides* against *D. maculatus,* as well as the local availability of the plant make it an attractive choice in pest-management practices. Therefore, dry fish traders are advised to use *A. conyzoides* for the protection and storage of smoked-dry fish against weevil infestations.

Keywords: Insecticidal, Toxicity, Mortality, Weevil, *Dermestes maculatus, Ageratum conyzoides*

1. Introduction

Smoked fish is one of the most widely distributed and cheapest animal protein product available in Nigeria. It is also an important source of food and income for many people, especially in the Southern part of Nigeria, where over 25% of the population depend on it as a rich source of protein, essential amino acids, vitamins and minerals (Azam *et al.,* 2004; Aderolu and Akpabio, 2009 and Ito, 2017). In West Africa, particularly Nigeria; the total annual consumption of fish is 1.2 million tons (FAO, 2004 and FDF, 2005) of which 45% of the total fish catch are utilized as smoked fish (FAO, 2002).

In Nigeria and most West African countries, the most common pest of animal products, which also infest smoked fish, is the hide weevil *Dermestes maculatus* (Degeer). A large-scale deterioration in quality, and 50% losses in quantity of dried fish, due to dermestid infestation, have been reported (Egwunyenga *et al.,* 1998 and Odeyemi *et al.,* 2000). Ito and Ighere (2017a) stated that during storage, transportation and marketing, smoked fish is readily attacked by several species of insects, notably *Dermestes maculatus, D. frischii, D. ater* and *Necrobia rufipes*. These weevils form aggregations of 1 – 13 weevils around food sources where individuals feed and mate (McNamara *et al.,* 2008).

Problems of pest resistance/resurgence, residual/ vertebrate toxicity, widespread environmental hazards, and the increasing costs of the application of synthetic insecticides have created a need for the utilization of effective, ecofriendly and biodegradable botanicals such as *Ageratum conyzoides* (goat weed), a promising botanical insecticide. The use of plant products in the control of insect pests is influenced by its availability, safety and effectiveness. *A. conyzoides* is a common weed found in several countries in tropical and sub-tropical regions, including Nigeria where its control is often difficult. The aqueous extracts of the whole plant have been used by Shabana *et al.,* (1990) to cause a significant reduction of larvae emergence of root knot nematode, *Meloidogyne incognita*. Gbolade *et al.,* (1999) also confirmed the

* Corresponding author. e-mail: ito.eddie@yahoo.com.

insecticidal properties of the volatile oil of *A. conyzoides* against *Callosobruchus maculatus* and *Tribolium cataneum* (Singh *et al.,* 2014). The aqueous extracts of the leaves or whole plants have been used to treat colic, colds and fevers, diarrhea, rheumatism, spasms; they have been used also as a tonic (Negrelle *et al.,* 1988; Oliveira *et al.,* 1993). *A. conyzoides* contains monoterpenoids, diterpenoids, sesquiterpenoids and other compounds, including alkaloids, cumarins, flavonoids, chromenes (conyzorigum), benzofurans, sterols and terpenoids, which exhibit ovicidal, larvicidal, repellent, deterrent, antifeedant, and toxic effects in a wide range of insects. This study is aimed at evaluating the bioinsecticidal potentials of the leaf powder and extracts of goat weed, (*A. conyzoides*), on fish weevils (*Dermestes maculates)*.

2. Materials and Methods

2.1. Preparation of Plant Powders

The leaves of *A. conyzoides* were dried under shade for several days to prevent breakdown and loss of phytochemical components. The dry leaves were ground by an electric blender and were sieved to obtain the powder which was stored in an air tight container.

2.2. Preparation of Crude Extracts

The extract of the *A. conyzoides* plant material was prepared by dissolving 50 g of the powder in 1000 mL of 95% ethanol, giving a concentration of 0.05 g/mL each. The extraction was done using the Soxhlete extractor. The process was repeated several times, and the supernatant of the plant extracts were decanted. A crude extract was obtained after the complete removal of the solvents with vacuum evaporation at temperature <40ºC to produce a thick liquid and syrupy material. From the main extract, 1.5 g, 2.25 g and 3 g were taken and dissolved in 30 mL of the solvent in a separate jar to produce a concentration of 50 mg^{-ml} 75 mg^{-ml} and 100 mg^{-ml} extract concentration which was used for the test.

2.3. Insect Culture and Maintenance

The *Dermestes maculatus* weevils used in this study were obtained from infested catfish (*Clarias gariepinus*) bought locally from Abraka market in Delta State, Nigeria. The pest was cultured in the Department of Animal and Environment Biology Laboratory, Delta State University, Abraka. Heavily infested catfish were put in different plastic containers covered with muslin cloth, and held tightly in a place to prevent the entry and exit of the weevils. A new generation of *D. maculatus* was obtained from the stock cultured by infesting clean uninfected catfish with adult *D. maculatus*. The newly emerged insects were then collected and used for the study (Egwuyenga *et al.,* 1998).

2.4. Toxicity Test of A. conyzoides Powders

The current study was carried out using four different doses (0.5, 1, 1.5, 2, 2.5 and 3 g) of *A. conyzoides* powders. Each dose was placed in a clean Petri dish and replicated three times. Ten grams of uninfested dry catfish (*C. gariepinus*) were put into the different Petri dishes containing the plant powder. This was done in the replicate of the different doses. Each Petri dish was shaken mechanically to ensure that the powder and dry catfish were thoroughly mixed. Twenty unsexed adult weevils were collected from the culture, and added to each treated catfish in the Petri dishes. The dishes were then covered to prevent the weevils from escaping. A control experiment consisting of ten grams of catfish and twenty weevils was also setup and replicated. The experimental set-up was observed for pest mortality over a period of 120 hours (5 days).

2.5. Toxicity Test of Crude Plant Extracts of A. conyzoides

This study was carried out using three different concentrations (50, 75, and 100 mg^{-ml}) of the *A. conyzoides* leaf extract. Each concentration (50, 75 and 100 mg^{-ml}) was used to treat the filter paper placed on a clean Petri dish, and was left for twenty-four hours to dry. These were replicated three times for each concentration. Ten grams of uninfected dry catfish (*C. gariepinus*) were put into the different Petri dishes containing the plant extract. This was done in the replicate of the different concentrations. Twenty unsexed adult weevils (*D. maculatus*) were collected from the culture and added to each treated Petri dish. The dishes were then covered to prevent the escaping of the weevils. A control experiment consisting of ten grams of catfish and twenty weevils was also setup and replicated. The experimental set-up was observed for a period of seventy-two hours for pest mortality.

2.6. Statistical Analysis

The data collected were analyzed by finding the average number of dead *D. maculates* during the 120-hour exposure to the different concentrations, and then converting it to mortality percentage. The mean percentage mortality obtained was then subjected to a two-way ANOVA to determine the significant difference using SPSS 17, and the results were interpreted accordingly.

3. Results

The results of the assessment of the biopesticidal efficacy of *A. conyzoides* (goat weed) at different concentrations of powders and extracts against *D. maculatus* (fish weevil) over a 120-hour period of exposure are presented in Tables 1 – 4.

3.1. Toxicity of A. conyzoides Powder on D. maculatus

Table 1 shows that the daily (24 h interval) mean mortality of the unsexed catfish weevils under the effect of the *A. conyzoides* powder over a 24 – 120-hour exposure period gave a cumulative mean percentage mortality of 43.33, 76.67, 88.33, 96.67, 98.33 and 100% at 0.5, 1, 1.5, 2, 2.5, and 3 g concentrations respectively. This study also showed that mortality increased as the gram (g) concentration of the powder of *A. conyzoides* increased from 0.5 – 3.0 g. Statistically, this indicated that the application of the *A. conyzoides* powder with different concentrations had a significant ($P < 0.05$; $F = 13.69$) effect on the mean percentage mortality of *D. maculatus* over a 120-hour period of exposure. The data presented in Table 1 revealed also that the *A. conyzoides* powder at its peak concentration (3.0 g) gave the highest mortality (100 %) of fish weevils. The first twenty-four hours of application of the plant powder recorded the highest weevil mortality with all the concentration of *A.*

conyzoides (Table 1). However, no mortality was observed in the negative control (triplicates without plant treatment).

The results of the probit analysis percentage for the median lethal concentration (LC_{50}) on the mortality of *D. maculatus* is presented in Figure 1. The minimum concentration required to kill 50% of the fish weevils was determined to be a concentration of 0.59 g (Table 1).

3.1.1. Median Lethal Time LT_{50} of A. conyzoides Powder

The respective regression equation, R^2 and LT_{50} values caused by the plant powders at different concentrations are presented in Table 2. The minimum time required to kill 50% of *D. maculatus* at 0.5 – 3.0 g of *A. conyzoides* was determined (Figures 2 – 7). The LT_{50} value for gram concentration after the treatment indicated that 3.0 g was the most toxic at the minimum time giving an LT_{50} of 22.80 h (Table 2). 0.5 g was the least effective dose causing a mortality rate of 50% of *D. maculatus* over an exposure time of 115.2 hours.

Table 1. Mean percentage mortality (Mean ± S.E) of *D. maculatus* exposed to *A. conyzoides* leaf dust in 120 hours.

Plant powder Doses (g)	Mean Mortality (%)					No. of Dead weevil	Cumulative Mean % Mortality at 120h	LC_{50} (g)
	Exposure Hours (h)							
	24h	48h	72h	96h	120h			
Control	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00	0.00	0.59
0.5	11.67 ± 1.67	11.67 ± 1.67	10.00 ± 0.00	6.67 ± 1.67	3.33 ± 1.67	26.00	43.33	
1.0	25.00 ± 2.89	18.33 ± 1.67	15.00 ± 2.89	10.00 ± 2.89	8.33 ± 3.33	46.00	76.67	
1.5	30.00 ± 2.89	25.00 ± 5.00	18.33 ± 4.41	8.33 ± 4.41	6.67 ± 1/67	53.00	88.33	
2.0	28.33± 6.01	25.00 ± 5.77	25.00 ± 2.89	13.33 ± 4.41	5.00 ± 2.89	58.00	96.67	
2.5	31.67 ± 1.67	28.33 ± 3.33	30.00 ± 5.77	5.00 ± 5.00	3.33 ± 3.33	59.00	98.33	
3.0	41.67 ± 4.41	26.67 ± 4.41	25.00 ± 2.89	6.67 ± 6.67	0.00 ± 0.00	60.00	100.00	

Percentage values are mean of triplicates observations with 20 weevils per replicate; F = 13.69; P<0.05

Table 2. Cumulative mean mortality (%) of *D. maculatus*, regression equation and median lethal time (LT_{50}) caused by *A. conyzoides* leaf dust.

Plant powder Doses (g)	Cumulative Mean Mortality (%)					Regression equation	R^2 value	Correlation (%)	LT_{50} (h)
	Exposure Hours (h)								
	24h	48h	72h	96h	120h				
0.5	11.67	23.34	33.34	40.01	43.33	y = 0.4266x + 1.12	0.9264	92.64	115.2
1.0	25.00	43.33	58.33	68.33	76.67	y = 0.5347x + 15.83	0.9732	97.32	64.20
1.5	30.00	55.00	73.33	78.33	88.33	y = 0.5972x + 22.67	0.9277	92.77	45.60
2.0	28.33	53.33	78.33	91.66	96.67	y = 0.7291x + 17.17	0.9384	93.84	45.60
2.5	31.67	60.00	90.00	95.00	98.33	y = 0.7013x + 24.50	0.8659	86.59	36.00
3.0	41.67	68.34	93.34	100.00	100.00	y = 0.618x 36.17	0.8524	85.24	22.80

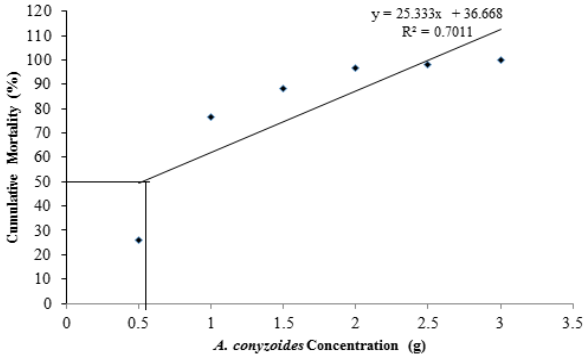


Figure 1. Percentage probit kill of *D. maculatus* exposed to *A. conyzoides* gram concentration at

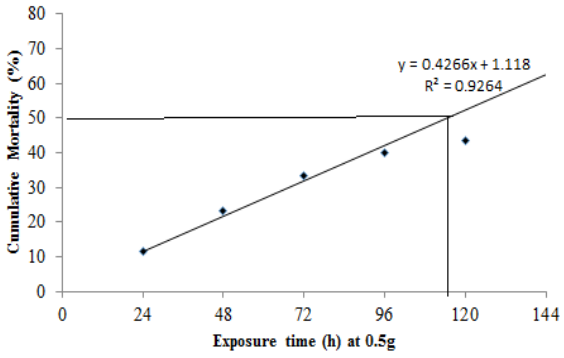


Figure 2. Time-mortality (LT_{50}) response of *D. maculatus* exposed to 0.5 g of *A. conyzoides* dust.

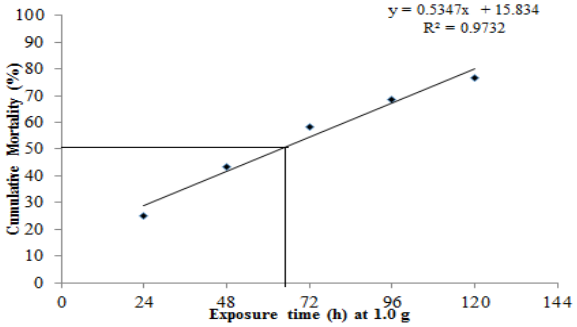


Figure 3. Time-mortality (LT_{50}) response of *D. maculatus* exposed to 1.0 g of *A. conyzoides* dust.

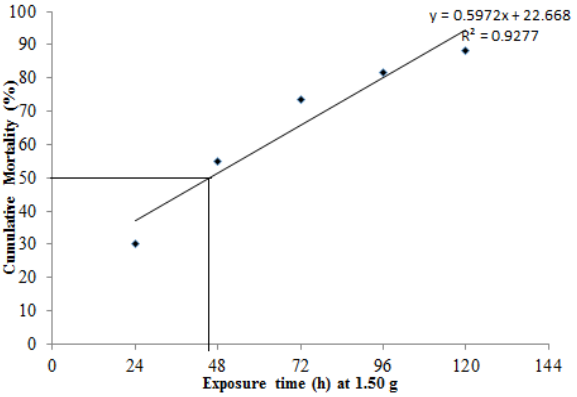


Figure 4. Time-mortality (LT_{50}) response of *D. maculatus* exposed to 1.5 g of *A. conyzoides* dust.

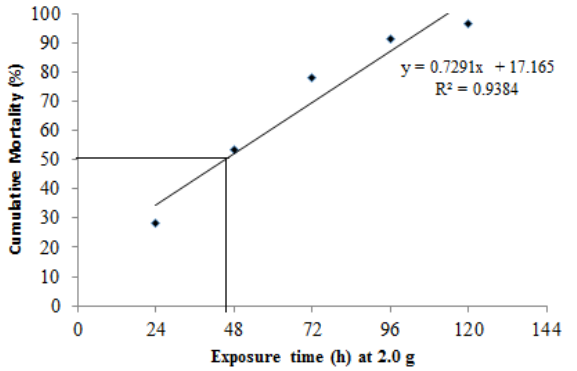


Figure 5. Time-mortality (LT_{50}) response of *D. maculatus* exposed to 2.0 g of *A. conyzoides* dust.

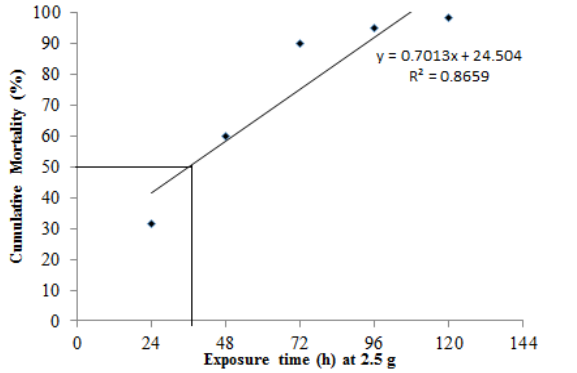


Figure 6. Time-mortality (LT_{50}) response of *D. maculatus* exposed to 2.5 g of *A. conyzoides* dust.

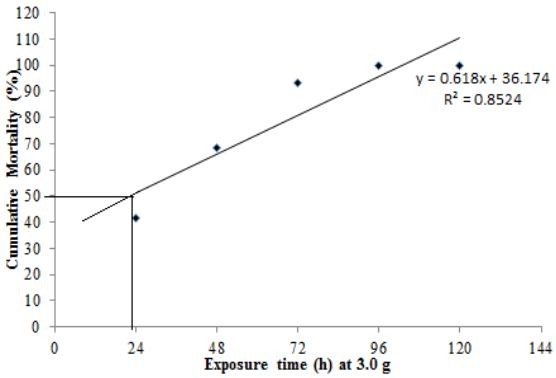


Figure 7. Time-mortality (LT_{50}) response of *D. maculatus* exposed to 3.0 g of *A. conyzoides* dust.

3.2. Toxicity of A. conyzoides Extract on D. maculatus

The mortality caused by *A. conyzoides* also showed a similar mortality trend similar to the powder. Here, mortality of *D. maculatus* increased with increasing the concentration (mg-mL) of the extract used in the test; hence the mortality was concentration-dependent. The extract of *A. conyzoides* at 75.0 and 100.0 mg-mL per ten grams of smoked catfish exhibited the highest cumulative mean mortality of 93.34 % and 100.0% respectively (Table 3). ANOVA showed that the application of *A. conyzoides* extract at different concentrations had no significant effect ($P > 0.05$) on the mean percentage mortality of *D. maculatus* over a seventy-two-hour period of exposure ($P = 0.2573$; $F = 1.7422$). The results for the probit analysis percentage for median lethal concentration (LC_{50}) on the mortality of *D. maculatus* is presented in Figure 8. The minimum concentration required to kill 50% of the fish weevils was determined to be an extract concentration of 36.86 mg-ml (Table 3).

3.2.1. Median Lethal Time LT_{50} of the A. conyzoides Extract

The regression equation, (R^2 and LT_{50}) values caused by the leaf extract of the plant at different concentrations are presented in Table 4. The minimum time required to kill 50% of *D. maculatus* at 50.0 – 100.0 mg-mL of *A. conyzoides* was also determined and presented in Figures 9 – 11). The LT_{50} value for all the extract concentrations after the treatment indicated that 100.0 mg-mL was the most toxic with the minimum time giving an LT_{50} of 15.40 h (Table 4). The least effective dose was 50.0 mg-mL, causing 50% mortality of *D. maculatus* after 26.48 hours of exposure.

Table 3. Mean percentage mortality (Mean ± S.E) of *D. maculatus* exposed to *A. conyzoides* leaf extract in 72 hours.

Plants' Extract Conc. (mg/mL)	Mean Mortality (%)			No. of Dead weevil	Cumulative Mean % Mortality at 120h	LC_{50} (mg^{-ml})
	Exposure Hours (h)					
	24h	48h	72h			
Control	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00	0.00	36.86
50.0	43.33 ± 0.33	33.33 ± 0.33	13.33 ± 0.33	54.00	89.99	
75.0	45.00 ± 0.58	31.67 ± 0.33	16.67 ± 0.33	56.00	93.34	
100.0	53.33 ± 0.33	36.67 ± 0.88	10.00 ± 0.58	60.00	100.00	

Percentage values are mean of triplicates observations with 20 weevils per replicate; F = 1.7422; $P > 0.05$

Table 4. Cumulative mean percentage mortality of *D. maculatus*, regression equation and median lethal time (LT_{50}) caused by *A. conyzoides* leaf extract**.**

Plants' Extract Conc. (mg/mL)	Cumulative Mean Mortality (%)			Regression equation	R^2 value	Correlation (%)	LT_{50} (h)
	Exposure Hours (h)						
	24h	48h	72h				
50.0	43.33	76.66	89.99	y = 0.9721x + 23.33	0.9423	94.23	26.48
75.0	45.00	76.67	93.34	y = 1.0071x + 23.33	0.9689	96.89	26.00
100.0	53.33	90.00	100.00	y = 0.9723x + 34.44	0.9018	90.18	15.40

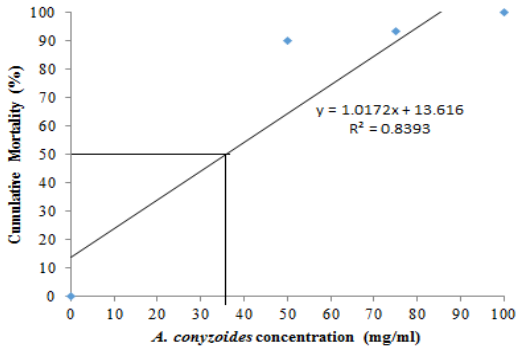


Figure 8. Percentage probit kill of *D. maculatus* exposed to *A. conyzoides* extract concentration at LC_{50}; Regression equation inclusive.

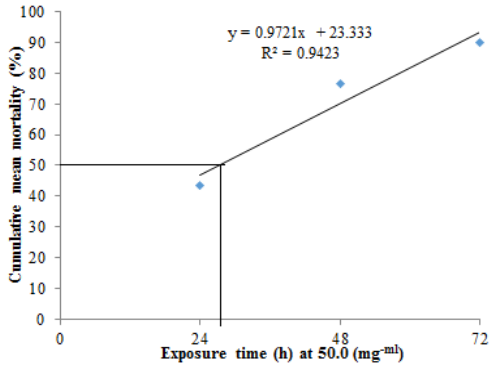


Figure 9. Time-mortality (LT_{50}) response of *D. maculatus* exposed to 50.0 mg^{-ml} of *A. conyzoides* extract.

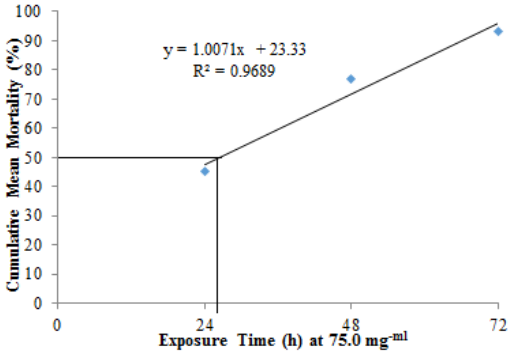


Figure 10. Time-mortality (LT_{50}) response of *D. maculatus* exposed to 75.0 mg^{-ml} of *A. conyzoides* extract.

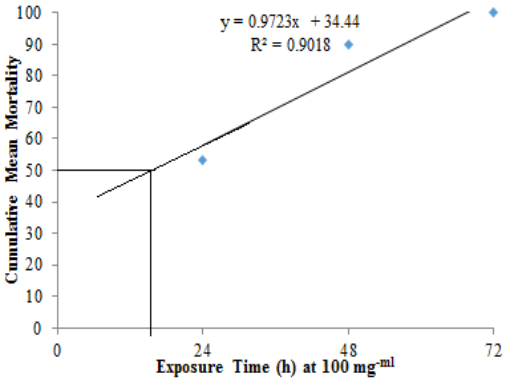


Figure 11. Time-mortality (LT_{50}) response of *D. maculatus* (fish weevil) exposed to 100.0 mg^{-ml} of *A. conyzoides* extract.

4. Discussion

The current study showed that the *A. conyzoides* powder treatment was toxic to *D. maculates,* and the application of the powder at different concentrations had a significant effect ($P < 0.05$) on the mean percentage mortality of the weevils over a 120-hour period of exposure. A high mortality rate of 100.0% of the weevils was recorded at 3.0 g of the *A. conyzoides* powder per ten grams of the substrate (dry smoked catfish). This finding is in agreement with Singh *et al*., (2014) who reported a 100.0% mortality rate of *T. castaneum* using *A. conyzoides.* Similarly, Akinwumi (2011) also documented a 100.0% rate killing *D. maculatus* with ten grams of powder per 100.0 g of fish using *Dennettia tripetala*, *Eugenia aromatic, Piper guineense,* and *Monodora myristica*. The current study also revealed that the minimum concentration required to kill 50% of the fish weevils was 0.59 g of the plant/10.0 g of smoked-dried fish (Table 1).

In the present study, there was no significant difference (P>0.05) in the percentage of mortality of *D. maculatus* using *A. conyzoides* extracts as shown by the statistical analysis. In all treatments, mortality was relatively more at higher doses (2.5 g and 3.0 g), and more with the extract concentration being (75 and 100 mg^{-ml}) than lower extract concentration (50 mg^{-ml}). This shows that *A. conyzoides* has promising bioactive properties for dry-smoked fish traders in the tropics. The crude plant extracts of the plant have also showed insecticidal and pesticidal activities against various types of insects and pests (Kamboj and Saluja 2008). The application of the ethanol extract and powder on *D. maculatus* exhibited high insecticidal activity against the weevils (Table 3). The findings of the current study are in accordance with those of other researchers, who had reported earlier that the plant powders and extracts, including those of *A. conyzoides* are toxic to insects (Singh *et al.,* 2014; Ito *et al.,* 2015; Uwamose *et al.,* 2017 and Ito and Ighere 2017b). The mortality percentage was significantly ($P < 0.05$) higher with the use of the powder of *A. conyzoides,* but not the ethanolic extract ($P > 0.05$). The extracts caused more than 80% mortality rate at all extract concentrations. Although no mortality was observed in the control set-up. The results obtained in this study are in accordance with Ito and Ighere (2017b) who reported that ethanolic extracts of the plant usually contain more active insecticidal ingredients than the powder.

The extract of *A. conyzoides* gave better mortality effects than the leaf powder (Table 1 and 3). The different effects may can be attributed to the presence of higher proportions of active chemical components in the extract than in the powder. A wide range of chemical compounds, including alkaloids, cumarins, flavonoids, chromenes, benzofurans, sterols, and terpenoids have been isolated from *A. conyzoides*. Extracts and metabolites from this plant have been found to possess pharmacological and insecticidal activities (Vyas and Mulchandani, 1980). *A. conyzoides* contain bioactive compounds, such as terpenics, mainly precocenes, with antijuvenile hormonal activity that may affect the growth and development of the insect, rather than being the direct cause to insect mortality. Vyas and Mulchandani (1980) reported the action of chromenes (precocenes I and II) isolated from *Ageratum* plants that accelerate larval metamorphosis resulting in maintaining the juvenile forms or producing weak and small adults. They also stated that conyzorigum is the active ingredient of chromene in *A. conyzoides* which could be the reason for the high mortality observed in this present study. The two chromenes have been reported to act synergistically, and they survived metabolism for at least twelve days (Fagoonee and Umrit, 1981).

Comparatively, the mortality of weevils fed on fish substrate, and treated with the extract of the *A. conyzoides* leaf at both concentrations was very much higher than those fed on substrate and treated with the powder. This indicates that the leaf powder of *A. conyzoides* has less bioactive substances than the extract. The cause of the high mortality of *D. maculatus* adults within three to five DAT could be due to conyzorigum substances in *A. conyzoides* which act as an antifeedant and a stomach poison. There is a possibility that the active component, conyzorigum, whose synergetic effects of precocenes I and II was at the highest concentration in the extract resulting in rapid mortality of adult *D. maculatus*.

5. Conclusion

The use of indigenous plant-based products by individuals and communities can provide prophylactic measures for the protection against various insect pest infestations. Therefore, smoked-fish traders are advised to use the *A. conyzoides* plant, commonly called "shell leaf, in Nigeria, for the better protection and storage of their products, because of its effectiveness, less hazards, availability and easy accessibility, and also for its medicinal functions. The ethanolic extract of *A. conyzoides* is a better botanical insecticide; however, further studies need to be conducted to ascertain which phytochemical is the active ingredient responsible for the *D. maculatus* mortality.

Acknowledgment

We would like to thank the Administration at Delta State University in Abraka, particularly Dr. J.E.G Ake, of the Department of Animal and Environmental Biology for his constructive criticism. We also thank the Tropical Disease Research (TDR) Unit and the Center for Research in Environmental Resources Management (CREMA) for providing facilities, research grants, and enabling environments for this research.

Conflicts of interest: Nil

References

Aderolu AZ and Akpabio VM. 2009. Growth and economic performance of *Clarias gariepinus* juveniles fed diets containing velvet bean, *Mucuna pruriens*, seed meal. *Afr J Aquat Sci*., **34**: 131-135.

Akinwumi FO. 2011. Evaluation of Some Plant Materials for the Control of Smoked Fish Pest, *Dermestes maculatus* DEGEER (Coleoptera: Dermestidae) in *Clarias gariepinus* Burchell (Pisces: Clariidae). *ARPN J Agric & Biol Sci*, **6**(7); 65 – 69.

Azam K, Ali MY, Asaduzzaman M, Basher MZ and Hossain MM. 2004. Biochemical assessment of selected fresh fish. *J Biol Sci*, **4**: 9-10.

Egwunyenga OA, Alo EB and Nmorsi OPG. 1998. Laboratory evaluation of the repellency of *Dennettia tripetala* Baker (Anonaceae) to *Dermestes maculatus* (F) (Coleoptera: Dermestidae). *J. Stored Product Res.,* **34**: 195-199.

Fagoonee I, and Umrit G. 1981. Antigonadotropic hormones from the goat weed, *Ageratum comyzoides*. *Insect Sci Appl.,* **4**: 373-6.

FAO. 2002. Yearbook fishery statistics. Food and Agric. Organiz., 92/2, pp: 186.

FAO. 2004. The State of World Fisheries and Aquaculture (SOFIA) PART 1: World Review of Fisheries and Aquaculture. Food Agric. Organiz, Fish. Rep., Rome.

FDF. 2005. Federal Department of Fisheries. Federal Ministry of Agriculture and Rural Development Report of Presidential Committee on Fisheries and Aquaculture Development, pp: 1, 63.

Gbolade AA, Onayade OA, Ayinde BA. 1999. Insecticidal activity of *Ageratum conyzoides* L. volatile oil against *Callosobruchus chinensis* F in seed treatment and fumigation laboratory tests: Insect Science and its application. *Insect Sci Its Appl,* **19**:237.

Ito EE. 2017. Survey of parasites of two fish species (*Tilapia zillii* and *Clarias gariepinus*) in Ase River Catchment, Delta State, Nigeria. *J Coastal Life Med,* **5**(10): 417-421.

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

Ito EE and Ighere EJ. 2017b Bio-insecticidal potency of five plant extracts against cowpea weevil, *Callosobrichus maculatus* (F., 1775), on stored cowpea, *Vigna unguiculata* (L). *Jordan J Biol Sci,* **10**(4):

Ito EE, Nmor JC, Ake JEG and Uteboe K. 2015. Larvicidal activities of *Pistia stratiotes* (water lettuce) against 3rd and 4th instar larvae of *Aedes aegypti*. *Adv Res*, **3**(6): 589-595.

Kamboj A, and Saluja AK. 2008. *Ageratum conyzoides (*L.): A review on its phytochemical and pharmacological profile. *Int J Green Pharm*, **2**:59-68.

McNamara KB, Brown RL, Elgar MA, and Jones TM. 2008. Paternity costs from polyandry compensated by increased fecundity in the hide beetle. *Behavioral Ecol*, **19**: 433-440.

Negrelle RRB, Sbalchiero D, and Cervi AC. 1988. Espécies vegetais utilizadas na terapêutica popular no município de Curitiba, Paraná, Brasil. Univ. Federal do Paraná.

Odeyemi OO, Owoade RA and Akinkurolere O. 2000. Toxicity and population suppression effects of *Parkia clappatoniana* on dried fish pests (*Dermestes maculatus* and *Necrobia rufipes*). *Global J Pure and Applied Sci*., **6**: 191-195.

Oliveira F, MK, Akisue and Garcia LO. 1993. Caracterização farmacognóstica da droga e do extrato fluído de mentrasto, *Ageratum conyzoides* L. *Lecta* **11**(1):63–100.

Shabana N, Husain SI, and Nisar S. 1990. Allelopathic effects of some plants on the larval emergence of *Meloidogyne incognita*. *J. Indian Appl. Pure Biol.* **5**:129–130.

Singh JP, Prakash B, and. Dubey NK. 2014. Insecticidal activity of *Ageratum conyzoides* L., Coleus aromaticus Benth. and *Hyptis suaveolens* (L.) Poit essential oils as fumigant against storage grain insect *Tribolium castaneum* Herbst. *J Food Sci Technol*., **51**(9): 2210–2215.

Uwamose MO, Nmor JC, Okologho BC, Ake JEG. 2017. Toxicity of lemon grass *Cymbopogon citratus* powder and methanol extract against rice weevil *Sitophilus oryzae* (Coleoptera: Curculionidae). *J Coastal Life Med*, **5**(3): 99 – 103.

Vyas AV, Mulchandani NB. 1980. Biosynthesis of procoenes-I and II antijuvenile hormones. *Phytochem*, **19**: 2597-2598.

JJBS

Volume 11, Number 2,June 2018
ISSN 1995-6673
Pages 231 - 233

Jordan Journal of Biological Sciences

Short Communication

Surgical Site Infections in a Tertiary Referral Hospital in Amman: Causative Bacteria and Antibiotic Susceptibility

Mahmoud M. Al-Awaysheh*

Department of General Surgery, Faculty of Medicine, Mutah University, P.O.Box 7, Mutah 61710, Al-Karak, Jordan

Received October 17, 2017; Revised November 5, 2017; Accepted November 11, 2017

Abstract

Surgical site infections (SSIs) represent a major healthcare challenge. This retrospective study aimed to assess the frequency of SSIs, the bacterial profile, and the antibiogram of the isolates, from a tertiary hospital in Jordan. Data regarding SSIs were obtained from hospital records throughout the year 2015. The prevalence rate of SSIs was 5.4%. Gram-negative bacteria were more common than Gram-positive (57% vs. 43%). *Staphylococcus aureus* (35%) and *Escherichia coli* (24.5%) were the most common etiologies. Among Gram-positive isolates resistance was highest for ampicillin, and least for linezolid, teicoplanin, and vancomycin. Among Gram-negative isolates, resistance was highest for ampicillin, ciprofloxacin, and amoxicillin-clavulanate, and least for gentamicin, piperacillin-tazobactam, and meropenem. Surveillance of SSIs, the causative bacteria, and the bacterial antibiograms, are necessary for implementing strict infection control measures, and in the selection of effective antibiotic treatments to decrease the mortality and morbidity rates associated with SSIs. Most SSI cases were detected in individuals aged fifty-five years old or more, and combined with comorbidities, ex. diabetes mellitus.
Keywords: Surgical site infection, Prevalence, Antibiogram, Jordan, *S. aureus, E. coli*.

1. Introduction

Surgical site infections (SSIs) represent a major part of the health-care associated infections worldwide, and are responsible for the increasing morbidity and mortality rates, length of hospital stay, and health-care costs (Owens and Stoessel, 2008).

Despite advances in infection control and surgical procedures, SSIs are considered a serious healthcare problem with an estimated incidence rate of 2-20%, even in modern hospital settings (Hohmann *et al.,* 2012; Owens and Stoessel, 2008; Vikrant *et al.,* 2015). For example, SSI incidence rates in different socioeconomic level countries were 20.3% in Nigeria, 16% in India, and 14.7% in Japan (Qasem and Hweidi, 2017).

SSIs can be attributed to endogenous or exogenous bacteria during primary infections (infections detected, and causative agents isolated from the patients before starting the surgery). They may occur also as secondary infections after surgeries (Vikrant *et al.,* 2015). Multiple host and pathogen factors affect the risk of SSIs, including old age, existing co-morbidities, prolonged duration of surgery, and preoperative preparation and sterilization techniques (Masaadeh and Jaran, 2009; Owens and Stoessel, 2008).

Bacteriological causes of SSIs vary depending on factors such as geographical location, hospitals, wards, and type of surgical procedures (Owens and Stoessel, 2008). This can become more complicated in places where over-crowding, improper infection-control procedures, and antibiotic misuse exist. In particular, this is true in developing countries with low resources, where even basic operations such as appendectomies can lead to an increased mortality rate due to the high rates of infections (Vikrant *et al.,* 2015).

Surgical site infections are encountered in Jordan in healthcare settings associated with high rates of mortality and morbidity. For instance, SSI incidence in coronary artery bypass graft surgery (CABG) in Jordan reaching 16.8% (Qasem and Hweidi, 2017).

The aim of this study is to assess the bacterial profiles and the associated antibiotic susceptibility profiles in SSIs which occurred in a 450-bed tertiary hospital in Amman, the capital of Jordan.

2. Materials and Methods

This retrospective study was carried out in a tertiary hospital in Amman, Jordan. The hospital has a 450 bed capacity, and performs approximately 1900 surgeries per year.

The scientific approval for this study was obtained from Mutah University, Faculty of Medicine, Jordan. (19/5/2014)

Data on patients with confirmed SSIs for the year 2015 were collected from medical records. Data included age, sex, type of surgery, and presence of comorbidities.

* Corresponding author. e-mail: dr.Mahmoud60@yahoo.com.

The data on laboratory sample type, isolated species, and their antibiotic susceptibility, were collected electronically using Hakeem program (Electronic health solutions, 2014).

3. Results

A total of 102 cases were confirmed as SSIs constituting an overall incidence rate of about 5.4%. The mean age of patients was 55.1 years with a range of 25-75 years. Peak incidence was at ages older than fifty-five years. Males represented 46% of the cases with no statistically significant difference from females.

Isolated bacteria and their antibiograms are shown in tables 1 and 2 for Gram-positive and Gram-negative bacteria, respectively. Gram-positive bacteria (Table 1) were isolated from 44 (43%) cases, compared to 58 (57%) for Gram-negative bacteria (Table 2). *S. aureus* was the most commonly isolated species, and was found in 36 (35%) cases. Eight methicillin-resistant *S. aureus* (MRSA) isolates were also reported (7.8%). The second most-common species was *E. coli* which was found in 25 cases (24.5%). Other common bacteria included *Pseudomonas* spp. (15.6%) and *Citrobacter* spp. (9.8%).

The highest rate of resistance within the Gram-positive isolates was for ampicillin (75%) and ciprofloxacin (45.5%), but all isolates were susceptible to linezolid, teicoplanin, vancomycin, and cefoxitin (Table 1). The highest rate of resistance among Gram-negative isolates, was for ampicillin (87.2%), and the least was for meropenem (8.2%) (Table 2).

Patients with comorbidities, in particular patients with diabetes mellitus, were at a significantly higher risk of developing SSIs (data not shown).

Table 1. Antibiotic sensitivity for Gram-positive isolates.

Pathogen/count (Total 44)	Antibiotic susceptible isolates' count, %								
	AMP	CIP	SXT	GN	AMC	LNZ	TEC	VAN	CEF
S. aureus / 36	9, 25%	20, 55.5%	27,75%	22,61%	23, 63.8%	36, 100%	36, 100%	36, 100%	36, 100%
MRSA / 8	0	4, 50%	6, 75%	5, 62.5%	NT	8, 100%	8, 100%	8, 100%	8,100%

AMP: ampicillin, CIP: ciprofloxacin, SXT: trimethoprim sulfamethoxazole, GN: gentamicin, AMC: amoxicillin-Clavulanate, LZD: linezolid, TEC: teicoplanin, VAN, vancomycin, CEF: cefoxitin. NT: not tested.

Table 2. Antibiotic sensitivity for Gram-negative isolates.

Pathogen/count (Total 58)	Antibiotic susceptible isolates' count, %								
	AMP	CIP	CTX	SXT	GN	AMC	CAZ	TAZ	MEM
E. coli / 25	9, 36%	18, 72%	20, 80%	16, 64%	24,96%	17,68%	NT	25, 100%	25, 100%
Pseudomonas/ 16	0	4, 25%	NT	5, 31.2%	14, 7.5%	4, 25%	11, 8.7%	13,81.2%	15,93.7%
Citrobacter / 10	0	7, 70%	0	5, 50%	7, 70%	0	NT	7,70%	9, 90%
Proteus spp./ 4	1, 25%	2, 50%	2, 50%	2, 50%	3, 75%	3,75%	NT	3, 75%	3, 75%
Klebsiella / 3	0	2, 66.6%	1, 33.3%	2,66.6%	2, 66.6%	0	NT	2, 66.6%	3,100%

AMP: ampicillin, CIP: ciprofloxacin, CTX: cefotaxime, SXT: trimethoprim sulfamethoxazole, GN: gentamicin, AMC: amoxicillin-clavulanate, CAZ: ceftazidime, TAZ: piperacillin-tazobactam, MEM: meropenem. NT: not tested.

4. Discussion

Surgical site infections (SSIs) are still a challenging healthcare problem despite the advances in the surgical techniques, and the infection prevention measures (Owens and Stoessel, 2008; Vikrant *et al.,* 2015). The rate of SSIs varies from hospital to hospital with a range of 2.5% to 41.9% (Vikrant *et al.,* 2015; Malik *et al.,* 2011). In this study, the SSIs rate was 5.4%, which is consistent with what was previously reported (Malik *et al.,* 2011), but higher than SSIs rates in the United States (2.5%) and Europe (2-5%) (Satyanarayana *et al.,* 2011). Crowdedness and improper infection-control measures could explain the higher incidence in the current study compared to that in developed countries.

There was no significant difference regarding incidence between males and females, as they represented 46% and 54% of the cases, respectively. However, incidence rate increased with the increasing age, and the presence of comorbidities, mainly diabetes mellitus (data not shown). The mean age of patients was 55.1 years, and the peak incidence was at the age older than 55 years. Patients older than 55 years represented 60% of all SSIs cases. Advanced age and presence of underlying co-morbidities are important factors that increase the rate of SSIs due to conditions such as low immunity and slow healing wounds (Khan *et al.,* 2013).

In this study, the predominant species was *S. aureus* (35%), which is in agreement with previous studies that reported *S. aureus* to be the most common cause of SSIs (Chakarborty *et al.,* 2011; Mulu *et al.,* 2012). *S. aureus* is part of the normal flora and can lead to endogenous infections. However, healthcare workers, the environment, and contaminated instruments, may also be the source of such infections (Anguzu and Olila, 2007; Isibor *et al.,* 2008). All *S. aureus* isolates were susceptible to linezolid, teicoplanin, vancomycin, and cefoxitin, which is important to note when antibiotic guidelines are updated.

On the other hand, *E. coli* was the most common isolated Gram-negative species in this study with a rate of 23.1%, followed by *P. aeruginosa* at a rate of 14.8%. This is consistent to what has been reported previously by other studies (Ahmed, 2012; Anguzu and Olila, 2007; Mulu *et al.,* 2012; Vikrant *et al.,* 2015). Endogenous fecal flora could be the reason for the observed high incidence of *E. coli*, in addition to poor hospital hygiene (Malik *et al.,* 2011; Vikrant *et al.,* 2015). Susceptibility was highest for meropenem, and least for ampicillin.

The rate of SSIs in this study was comparable to that in other studies. However, the antibiogram was different than what was previously reported (Masaadeh and Jaran, 2009; Sohn *et al*., 2002; Vikrant *et al.,* 2015). This could be due to differences in the prevalence of bacterial strains among the population, variable infection-control measures and surgical techniques, and a high-likelihood of antibiotics misuse.

5. Conclusion

The findings of the current study give an additional significant insight into the SSIs prevalence and the antibiotic profile which are necessary for a better implementation of infection-control measures and antibiotic use guidelines. The findings create an awareness which would in turn decrease patients' mortality and morbidity rates, and provide guidance for the proper use of antibiotics and the implementation of strict infection-control measures. Studies with a bigger sample size and at a national level are recommended for more statistically relevant conclusions**.**

Acknowledgment

We would like to thank the Deanship of Academic Research at Mutah University for the financial support of this study. No. 248/14/120.

References

Ahmed MI. 2012. Prevalence of nosocomial wound infection among postoperative patients and antibiotics patterns at teaching hospital in Sudan. *N Am J Med Sci.,* **4:** 29-34.

Anguzu JR and Olila D. 2007. Drug sensitivity patterns of bacterial isolates from septic post-operative wounds in a regional referral hospital in Uganda. *Afr Health Sci.,* **7**: 148-154.

Chakarborty SP, Mahapatra SK, Bal M and Roy S. 2011. Isolation and identification of vancomycin resistant *Staphylococcus aureus* from postoperative pus sample. *Al Ameen J Med Sci*., **4**: 152-168.

Electronic health solutions, http://www.ehs.com.jo/en, accessed on December 2016.

Hohmann C, Eickhoff C, Radziwill R and Schulz M. 2012. Adherence to guidelines for antibiotic prophylaxis in surgery patients in German hospitals: a multicentre evaluation involving pharmacy interns. *Infection*, **40**: 131-137.

Isibor OJ, Oseni A and Eyaufe A. 2008. Incidence of aerobic bacteria and *Candida albicans*in postoperative wound infections. *Afr J Microbial Res.,* **2**: 288-291.

Khan AKA, Rashed MR and Banu G. 2013. A Study on the usage pattern of antimicrobial agents for the prevention of surgical site infections (SSIs) in a tertiary care teaching hospital. *J Clin Diagn Res.,* **7**: 671-674.

Malik S, Gupta A, Singh PK, Agarwal J and Singh M. 2011. Antibiogram of aerobic bacterial isolates from post- operative wound infections at a tertiary care hospital in India. *J Infect Dis Antimicrob. ,* **28**: 45-51.

Masaadeh HA and Jaran AS. 2009. Incident of *Pseudomonas aeruginosa* in post-operative wound infection. *Am J Infect Dis.*, **5**:1–6.

Mulu W, Kibru G, Beyene G and Datie M. 2012. Postoperative nosocomial infections and antimicrobial resistance patterns of bacterial isolates among patients admitted at Felege Hiwot Referral Hospital, Bahirdar, Ethiopia. *Ethiop J Health Sci.,* **22:** 7-18.

Owens CD and Stoessel K. 2008. Surgical site infections: epidemiology, microbiology and prevention. *J Hosp Infect.,* **70 (2)**: 3-10.

Qasem MN and Hweidi IM. 2017. Jordanian nurses' knowledge of preventing surgical site infections in acute care settings. *Open J Nursing*, **7**: 561-582

Satyanarayana V, Prashanth HV, Basavaraj B and Kavyashree AN. 2011. Study of surgical site infections in abdominal surgeries. *J Clin Diagn Res.*, **5**: 935-939.

Sohn AH, Parvez FM, Vu T, Hai HH, Bich NN and Le Thu TA. 2002. Prevalence of surgical-site infections and patterns of antimicrobial use in a large tertiary-care hospital in Ho Chi Minh City, Vietnam. *Infect Control Hosp Epidemiol.,* **23**: 382-387.

Vikrant N V, Pal SH, Juyal D, Sharma MK and Sharma N**.** 2015**.** Bacteriological profile of surgical site infections and their antibiogram: a study from resource constrained rural setting of Uttarakhand State, India. *J Clin Diagn Res.*, **9 (10)**: DC17-DC20.

JJBS

Volume 11, Number 2,June 2018
ISSN 1995-6673
Pages 235 - 240

Jordan Journal of Biological Sciences

Case Report

Mediterranean Spotted Fever and Early Neonatal Sepsis: the Transmission of *Rickettsia conorii* via the Placenta: A Case Report

Amjad S. Tarawneh[1,*], Omar A. Nafi[1], Zaid A. Samhan[2], and Khalaf A. Bani Hani[2]

[1] *Faculty of Medicine, Mutah University, Al-Karak,*

[2] *King Hussein Medical Center, Royal Medical Services, Amman, Jordan*

Received October 6, 2017; Revised October 30, 2017; Accepted November 4, 2017

Abstract

Mediterranean spotted fever (MSF) is a tick-borne disease caused by *Rickettsia conorii*. The classic clinical triad comprises high fever, an inoculation eschar, and a maculopapular non-pruritic rash. Serological testing is the most frequently used diagnostic method. Treatment involves antibiotics, preferably doxycycline. A two-day-old neonate developed sepsis and a skin rash. There was a high index of suspicion for MSF because the mother and four of her children were diagnosed with MSF in synchronization with the clinical presentation of this neonate. As far as one knows, congenital rickettsia as a cause of early neonatal sepsis has not been reported. However, limited data were reported using animal models. There are several challenges regarding its diagnosis and the selection of proper antibiotics for this age group. This paper discusses the challenges of its diagnosis, treatment, and impact on the neonate, this paper also reviews the literature regarding placental transmission of *Rickettsia conorii*. To the best of our knowledge, this is the first case study reporting that the early neonatal sepsis is caused by *R. conorii* (the Moroccan strain) in Jordan. Physicians caring for pregnant women and neonates living in areas with endemic MSF should consider the possibility of MSF in febrile patients and initiate early appropriate treatment based on a high index of suspicion.

Keywords: Mediterranean spotted fever; *Rickettsia conorii*; Neonate; Placenta; Doxycycline; Jordan

1. Introduction

Mediterranean spotted fever (MSF), also known as Boutonneuse fever, is an acute febrile zoonotic disease caused by *Rickettsia conorii*. It is transmitted to humans through a tick bite of the brown dog, *Rhipicephalus sanguineus*. It is one of the oldest recognized vector-borne infectious diseases (Parola *et al*., 2009). *R. conorii* is an obligate intracellular bacterium that infects vascular endothelial cells. MSF is an endemic disease present in many countries, especially those that surround the Mediterranean Sea. The first case of MSF was reported in 1910 in Tunisia; however, in the past few decades, the incidence of this tick-borne disease has remarkably increased in several countries (Rovery *et al*., 2008), including Jordan.

The clinical course of MSF is usually mild. However, it can be severe in some cases, and therefore requires special attention. The mortality rate of cases diagnosed with MSF has been estimated at approximately 2.5% (Bacellar *et al*., 2003). After an asymptomatic period of approximately one week following the infection, high fever usually arises with flu-like symptoms, and a black eschar at the tick-bite site usually occurs (Raoult and Roux**,** 1997). Between one to seven days (median, four days) following the onset of fever, a generalized maculopapular rash develops. Patients usually recover within ten days without any sequelae. However, severe forms of the disease are associated with different complications, including major neurological manifestations and multi-organ involvement in 5 to 6 % of the cases (Raoult, *et al*., 1983).

Diagnosis is usually based on the clinical symptoms and serology. In addition, common nonspecific laboratory abnormalities in rickettsiosis include mild leukopenia, anemia, and thrombocytopenia. Moreover, hyponatremia, hypoalbuminemia, and hepatic and renal abnormalities may occur, but testing for these non-specific abnormalities is not essential for establishing the diagnosis of MSF (Raoult *et al*., 1997). With respect to serological tests, a comparison of acute- to convalescent-phase serology is required; hence, it is only helpful in retrospect. To the best of the authors' knowledge, this is the first case report discussing the occurrence of early neonatal sepsis in Jordan as a result of the Mediterranean spotted fever (*R. conorii*; the Moroccan strain).

* Corresponding author. e-mail: amjadtarawneh@yahoo.com.

2. Case Presentation

The patient in the present case study was born in Al-Karak Hospital at a thirty-eight-week gestational age. The mother is twenty-nine years old, gravida 7, para 6. The results of her antenatal screening tests were as follows: rubella-immune; the venereal disease research laboratory test was nonreactive, hepatitis B serum antigen was negative, group B *Streptococcus* (GBS) status was unknown, and her blood type was B+. This pregnancy was uneventful until thirty-seven-week gestation (four days prior to admission) as the mother experienced fever, documented as 39°C when measured orally, together with dizziness, chills, diffuse skin rash, dry cough, and headache.

Her medical and surgical history was not relevant. Her family history revealed that four of her children had similar clinical profiles of high-grade fever, skin rash, and headaches. These children were admitted to the pediatric ward one day prior to her admission.

The general physical examination of the mother yielded normal results except for high-grade fever (39°C). However, the skin examination showed diffuse maculopapular rashes, which started four days prior to admission, and involved the entire body. The obstetric examination showed a thirty-eight-week single viable fetus in vertex presentation, with adequate amniotic fluid, anterior placenta, and no obvious gross congenital anomaly. The mother's other physical examinations were normal. Her basic investigations haven't revealed any abnormalities except for ESR which was 110 mm/hr.

The mother was admitted to the obstetrics ward with a chest infection, and was administered antibiotics (Ceftriaxone and Azithromycin). After three days of treatment, she was stable and afebrile. The non-stress test showed a normal reactive fetus. Induction of labor was initiated. Intrapartum prophylaxis for group B streptococcus was started using 2 g of ampicillin as the loading dose followed by 1 g every eight hours. The membranes were ruptured eight hours before delivery, and the liquor was clear. All stages of labour were uneventful. The newborn's APGAR score was 7/8, weight was 3 kg, and head circumference was 35 cm. The routine neonatal care was provided, and no specific resuscitation procedures were needed. Therefore, the newborn was discharged with his mother twelve hours after delivery following the routine postnatal examination.

The newborn was readmitted to hospital on day two of life with a history of high-grade fever associated with a maculopapular skin rash and diarrhea. In addition, he was hypoactive with poor feeding, and had been lethargic for twelve hours prior to admission. The general physical examination showed an ill-looking baby who was hypoactive but conscious and not distressed. The vital signs were as follows: temperature 39°C measured rectally; heart rate 140 bpm; Respiratory rate 60; O_2 saturation 96% in room air; blood pressure normal; and capillary refill time within two seconds. The newborn was hypoactive with weak crying, and jaundiced with acro-cyanosis, but no central cyanosis or pallor. The skin examination showed maculopapular skin rashes over the antecubital area, groin, and anterior aspect of the neck, which diffused later to involve the entire body. His neurological examination showed poor motor reflexes, poor sucking reflexes, and poor response to tactile stimulation, the anterior fontanelle was open and not bulging. All suture lines were normal, with no signs of birth trauma. There were no dysmorphic features. His cardiovascular, respiratory, and genitourinary examination results were normal.

The newborn was admitted for treatment of early neonatal sepsis. His initial septic work-up was negative for the usual-early neonatal sepsis-microorganisms (Table 1), the baby was administered ampicillin and gentamicin. A few hours after admission, he developed more diffuse skin rashes. The serological test results for his brother and sisters came back strongly positive for rickettsia (Table 2). Because the mother's serological test results were equivocally positive on day ten following the start of her symptoms, it was suspected that the newborn had congenital rickettsia. Therefore, he was administered doxycycline plus imipenem and vancomycin. His mother's serology for rickettsia was retested on day fourteen after the start of her symptoms, which again showed strongly positive results. The newborn's serological test results on days two and five after admission were negative, and on day ten they changed to weakly positive. The test used in this study was Vircell *Rickettsia conorii* plate: 96-wells plate coated with *R. conorii* antigen, strain Moroccan (ATCC VR-141) (Vircell S.L., Granada, Spain). The identification of the *Rickettsia* serotype (using the American Type Culture Collection VER-141) proved that it was *R. conorii* (the Moroccan strain) in all the positive cases of newborn's family. The newborn status improved forty-eight hours after admission. Fever subsided, skin rashes started to disappear, and his activities (including motor and sucking reflexes) significantly improved. He restarted feeding, and achieved full feeding on day nine of admission. The results of his laboratory examination for rickettsia-related complications were normal.

Table 1. Diagnostic data of the newborn

Laboratory	CBC : WBC, 15.5×10^3/µL	Hb, 18.5 g/dl
	Plt, 267×10^9/L	Mo, 8%
	Gr, 59	Lymph, 33%
	CSF: WBC, 2HPF; RBC, abundant traumatic tab; sugar and protein were normal.	
	Acute phase reactant: CRP: positive	
Cultures	No growth for blood, urine and CSF cultures	
Radiology	Brain CT: hyperdense tiny area seen in the left hemi-cerebellum may represent hemorrhage.	
	CXR: Normal	

CBC, complete blood count; RBC, red blood cell count; Hb, hemoglobin; WBC, white blood cell count; Plt, platelet; Mo, monocyte; Gr, granulocyte; Lymph, lymphocyte; CXR , chest X-ray; HPF, high-power field;

Table 2. Diagnostic Data of the Mother and the Siblings

Patient	Age (Years)	Presentation	LFT*	ESR*	Sodium*	Serology day 1	Serology day 7	Serology day 14
The mother	29	Fever, Skin rash	Normal	Elevated	decreased	Negative	Equivocal positive	Strongly Positive
Sibling 1	7	Fever, Skin rash	Slightly elevated	Elevated	decreased	Strongly Positive	Strongly Positive	Strongly Positive
Sibling 2	6	Fever, Skin rash	Slightly elevated	Elevated	decreased	Strongly Positive	Strongly Positive	Strongly Positive
Sibling 3	5	Fever, Skin rash	Slightly elevated	Elevated	decreased	Strongly Positive	Strongly Positive	Strongly Positive
Sibling 4	3	Fever, Skin rash	Slightly elevated	Elevated	decreased	Strongly Positive	Strongly Positive	Strongly Positive
The newborn		Fever, Skin rash	Slightly elevated	Elevated	Normal	Negative	Negative	Weakly positive

*LFT (liver function test), ESR (erythrocyte sedimentation rate) and sodium are positive tests usually been abnormal in Rickettsia infections

3. Discussion

3.1. Diagnosis and Work-up

The case presented here demonstrates the difficulties associated with the diagnosis and treatment of congenital MSF. The authors searched the medical literature in the PubMed database using the key words vertical transmission and trans-placental transmission, which were cross-matched with *R. conorii*, MSF, Boutonneuse fever, and rickettsiosis. Relevant references cited in the selected articles were also reviewed. During this search, we found very limited data describing congenital rickettsiosis in animal models. In consistence with the findings of these small publications, no vertical transmission was demonstrated. It has been demonstrated that rickettsiosis caused by *R. conorii* and *R. ricketsii* during pregnancy presented no risk of vertical transfer of these pathogens to offspring (Stallings, 2001**)**. However, the possibility of trans-placental transmission in other groups of pathogens, for example, *R. prowazekii*, transmitted by *P. humanus* in guinea pigs, has been demonstrated (Kurganova and Klimchuk, 1996). This small published data on animal models regarding congenital rickettsiosis due to *R. conorii* does not provide sufficient evidence supporting that the trans-placental transmission does not occur. Furthermore, this problem of trans-placental transmission of *R. conorii* needs to be additionally investigated in mammals, which can serve as reservoir species (Jasik *et al*., 2015).

Because congenital *R. conorii* infections are not well-reported for the neonatal age group, the diagnosis of MSF for this patient was not straightforward. The ultimate diagnosis was confirmed using his maternal history, family history, clinical presentation, and the epidemiological data of the disease in the study area.

3.2. Epidemiological Data

During the past few years, many cases of high-grade fever, skin rashes, and hyponatremia were observed. However, a black crusted eschar (tache noir), which is considered to be the site of the tick bite, was absent in these cases. Moreover, some of these cases were severe, and led to the development of thrombocytopenia and rapid multi-organ failure, resulting in a high mortality rate. Although comprehensive laboratory tests including polymerase chain reaction assays of several viruses were performed, no clear diagnosis could be made at the time. In the early summer of 2013, a small outbreak of cases occurred among patients all living in the same rural area and having close contacts with animals. Therefore, serological tests were performed for several diseases, including *Rickettsia*. In June 2013, the first case of *Rickettsia* was diagnosed at the formerly-mentioned hospital in Jordan. Since then, the same diagnosis has been given for each case presented with a high-grade fever and skin rash. In 2017, the authors published a retrospective observational study describing the epidemiological patterns of Mediterranean spotted fever (MSF), as well as its treatment and impact on children in southern Jordan (Nafi *et al*., 2017). Over a thirty-month period (from June 2013 to December 2015), we recorded fifty-five pediatric cases of MSF in that southern province, with an incidence rate of 7.9 cases per 100,000 inhabitants. MSF affected 89% of those individuals during summer. 74.5% of those patients were living in a rural area with tent housing, and 100% of them had contact with animals. All cases had fever, and 94.5% had a skin rash. Serological tests were positive in 87.2% of the cases. The incidence rate in Jordan is higher than that in Spain, but is lower than that in Bulgaria (Nafi *et al*., 2017).

The strong epidemiological link between this newborn and *R. conorii* was the history of his siblings and his mother, who were diagnosed and treated with *R. conorii* a few days before his birth. The diagnosis of *R. conorii* was confirmed by serological testing, and *R. conorii* (the Moroccan strain) was present in all the positive cases of his family.

3.3. Family History and Clinical Presentation

The clinical presentation of MSF in Jordan is similar to that in Europe (Nafi *et al*., 2017). Patients usually have a classic triad of fever and chills, severe headaches, and/or myalgia, in addition to a typical rash (Font-Creus *et al*., 1985; Raoult *et al*., 1986). The presence of a black crusted eschar (tache noir), which is considered to be the site of the tick bite, is very rare in MSF patients in Jordan (Nafi *et al*., 2017; Font-Creus *et al*., 1985; Raoult *et al*., 1986; Mumcuoglu and Keysary, 2002; Wolach *et al*., 1989).

The newborn of this study had three sisters and one brother who were admitted to the pediatric ward with the classic triad symptoms of MSF (fever and chills, severe headache, and myalgia). They also had a typical macular erythematous rash which appeared three days after the abrupt onset of fever. A black crusted eschar was absent in all the siblings. Based on the epidemiological data of the family and their clinical presentation, they were all admitted for suspicion of MSF, which was confirmed by the immunofluorescence assay on the same day of admission and by serotype testing on day seven after admission.

Although the clinical presentation of the mother at the disease onset was ambiguous, her diagnosis was established based on MSF findings in other family members and the epidemiological data of the family. Therefore, the mother was retested for *R. conorii* infection using the immunofluorescence assay on day two after the admission of her newborn (or day ten of her own disease onset). Results showed equivocal results initially, but they were strongly positive fourteen days after the disease onset, confirming that the mother had the Moroccan strain of *R. conorii*.

The newborn had a clinical profile similar to that of his family members, who were all (including the mother) confirmed to have MSF due to the infection with *R. conorii*. His initial septic work-up results were negative for the usual microorganisms. Because positive serology often requires waiting for convalescent titers, the decision was made to treat the patient for MSF due to *R. conorii* infection on day two of his admission. Nevertheless, diffuse skin rashes started to appear despite the fact that his mother's initial serological test results were equivocal. Most importantly, the clinical profile of *R. conorii* infection during early neonatal sepsis was not adequately studied, and there is very limited information in the published literature regarding its clinical presentation in the neonatal age group. Moreover, the early diagnosis and treatment of the disease are essential for an uncomplicated recovery. Because there are no convenient tests to diagnose the disease during its early course (Mumcuoglu and Keysary, 2002), the authors had to rely on a high index of suspicion despite the negative initial serology. As demonstrated in the mother's case, positive serology does require waiting for convalescent titers (Bentov *et al*., 2003).

3.4. Serologic Tests

Although the serologic tests are the most widely-available diagnostic tools for spotted fever, they are less than optimal for the diagnosis of rickettsia diseases' acute phases (Paddock et al., 1989). Moreover, the underdiagnosis of the fatal spotted fever may be attributed to the nonspecific clinical features and the insensitive acute-phase serologic studies (Weinberger *et al*., 2008). Serologic tests for the *R. conorii* infection in the newborn on day two of his admission (day ten of his mother's illness) yielded negative results (for both immunoglobulins IgM and IgG). On day ten of his illness, IgG results remained negative, and the IgM results were weakly positive. This may be attributed to the immature immune responses of neonates, or the fact that the mother was already treated with azithromycin, one of the alternative drugs for treating *R. conorii* infections during pregnancy (Bentov *et al*., 2003).

The importance of an early diagnosis and initiation of treatment in clinically-significant cases is reinforced by the fact that severe complications may develop, and the disease may unexpectedly take a rapid, fatal course. Fatalities have been reported in 1.4-5.6% of the hospitalized patients in Israel, France, and Portugal (Raoult *et al*., 1986; Mumcuoglu and Keysary, 2002; Aharonowitz *et al*., 1999; Botelho *et al*., 2012; Lucio-Villegas *et al*., 1990). Based on this, and due to the high index of suspicion, the diagnosis of MSF was made.

3.5. Treatment

In regions where MSF, caused by the *R. conorii* infection, is epidemic, an antibiotic therapy helps prevent the severe progression of the disease, and mortality (Lucio-Villegas *et al*., 1990; Anton *et al*., 2015). Although the clinical presentations and serological findings at the onset of disease in the patient were ambiguous, early treatment is considered to be essential for an uncomplicated recovery. Fatal outcomes in this region have been reported of previously- healthy children because the appropriate and timely antimicrobial drug-treatment was not administered in 2013 (Nafi *et al.*, 2017). Therefore, the administration of doxycycline pending the results of initial blood and CSF cultures, later showed no growth of the usual microorganism suspected during early neonatal sepsis.

The classic antibiotic therapy consists of tetracycline or chloramphenicol. The use of

Chloramphenicol in developed countries is limited due to its bone marrow toxicity (Bentov *et al*., 2003; Anton *et al*., 2015; Woodward 1991; Brouqui *et al*., 2007; Shaked *et al*., 1989). The first line of antibiotics for treating rickettsial diseases are tetracycline's (Bentov *et al*., 2003; Maurin and Raoult, 1997; Herbert *et al*., 1982). Doxycycline has replaced chloramphenicol as the drug of choice for this indication in children because of the recommendations of the American Academy of Pediatrics in 1991. Doxycycline has also become a common alternative for tetracycline, especially for children, because of its prolonged half-life enabling single or double daily doses, and because it is less prone to causing dental staining (Yagupsky *et al.*, 1987**)**. In 2015, Todd *et al* published a study indicating that children who received short-term courses of doxycycline at ages younger than eight years did not develop dental staining, enamel hypoplasia, or tooth color differences. That study provided the largest sample size and best evidence to date that short courses of doxycycline (such as those used to treat rickettsial diseases) do not cause dental staining when administered to children younger than eight years of age.

Both *in vitro* and *in vivo* studies have shown that doxycycline is highly efficacious for treating rickettsiosis (Anton *et al*., 2015). Even short-term treatments with doxycycline are highly effective (Anton *et al*., 2015; Bella-Cueto *et al*., 1987) Doxycycline is the gold standard treatment for MSF, and it is the most commonly used treatment for this disease (Anton *et al*., 2015). Clinical studies have demonstrated that doxycycline shortens the course of MSF and induces a rapid remission of symptoms (Botelho *et al*., 2012; Lucio-Villegas *et al*., 1990; Anton *et*

al., 2015). Recent evidence suggests that despite the potential side effects, doxycycline should be considered the drug of choice for children of all ages in whom a rickettsial disease is considered during the differential diagnosis of illness (Purvis and Edwards, 2000). Despite this, a national survey conducted in 2012 showed that only 35% of clinicians correctly chose doxycycline as the treatment of choice for suspected MSF in children younger than eight years old. The centers for disease control highlighted these issues in 2015. Doctors often avoid prescribing doxycycline to young children because of the warning that tooth staining may occur when used for children younger than eight years old. This misconception about the use of doxycycline for children prevented them from receiving a lifesaving treatment.

Based on previous evidence, doxycycline was administered to the patient of this study because it is believed to be the most effective treatment for all rickettsial diseases, and because previous studies have shown that other antibiotics are less effective, and are associated with a higher number of deaths (case fatality rate). The patient was discharged on day ten of admission, and was doing well, with full feedings achieved, afebrile status, and no skin rashes. He had regular follow-up examinations until he was eight months old, which showed that all of his growth parameters were within the normal ranges, including weight, body length, head circumference, hearing test, ophthalmic examination, and laboratory evaluations.

4. Conclusions

This case underscores the difficulties involved in establishing the diagnosis of MSF during pregnancy and the neonatal period. The absence of both, eschar and positive serology, which usually necessitates awaiting convalescent titers, is an obstacle to a correct diagnosis. For this reason, physicians caring for pregnant women and neonates living in areas with endemic MSF should consider the possibility of MSF in febrile patients and initiate early appropriate treatments based on a high index of suspicion. The suspicion could be attributed to the clinical presentation and the epidemiological data of the patient despite the negative initial serology. Doxycycline is the gold standard and most commonly-used treatment for MSF.

Based on the case of this study, R. *conorii* can assumingly penetrate the placental barrier and lead to the infection of the fetus and the newborn; this may be subject to evolutionary fitness. It has been shown through the review of literature that the mechanism for this pathogen to cross the placental barrier has been discussed in few model studies, but there is much ambiguity. For this reason, the issue of vertical transmission requires further attention.

Acknowledgments

We thank the directorate of the Disease Control Department and central laboratory of the Ministry of Health in Jordan. We also thank Editage (www.editage.com) for their English language editing services.

References

Aharonowitz G, Koton S, Segal S, Anis E and Green MS. 1999. Epidemiological characteristics of spotted fever in Israel over 26 years. *Clin Infect Dis*; **29**:1321-1322.

Anton E, Muñoz T, Travería FJ, Navarro G, Font B, Sanfeliub I and Segura F**.** 2015. Randomized trial of clarithromycin for Mediterranean spotted fever. *Antimicrob Agents Chemother* ; **60**:1642-1645.

Bacellar F, SousaR, SantosA, Santos-Silva M and Parola P. 2003. Boutonneuse fever in Portugal: 1995–2000. Data of a state laboratory. *Eur J Epidemiol*; **18**:275–277.

Bella-Cueto F, Font-Creus B, Segura-Porta F, Espejo-Arenas E, López-Parés P and Muñoz-Espín T. 1987. Comparative, randomized trial of one-daydoxycycline versus 10-day tetracycline therapy for Mediterranean spotted fever. *J Infect Dis* **155**:1056–1058.

Bentov Y, Sheiner E, Kenigsberg S and Mazor M. 2003. Mediterranean spotted fever during pregnancy: case presentation and literature review. *Eur J Obstet Gynecol Reprod Biol* ;**107**:214-216.

Botelho E, Socolovschi C, Raoult D and Parola P. 2012. Treatment of *Rickettsia* spp. infections: a review. *Expert Rev Anti Infect Ther*; **10**:1425–1437.

Brouqui P, Parola P, Fournier PE and Raoult D. 2007. Spotted fever rickettsioses in southern and Eastern Europe. *FEMS Immunol Med Microbiol*; **49**: 2–12.

Font-Creus B, Bella-Cueto F and Espejo-Arenas E. 1985. Mediterranean spotted fever: a cooperative study of 227 cases. *Rev Infect Dis*;**7**: 635-642.

Herbert WNP, Seeds J, Koontz WL and Cefalo RC. 1982. Rocky Mountain spotted fever in pregnancy: differential diagnosis and treatment. *South Med J*; **75**:1063–1066.

Jasik KP, Okła H, Słodki J, Rozwadowska B , Słodki A and Rupik W. 2015. Congenital tick borne diseases: Is this an alternative route of transmission of tick-borne pathogens in mammals?*Vector Borne Zoonotic Dis* ;**15**: 637-644.

Kurganova II and Klimchuk ND**.** 1996. Transplacental transmission of the causative agent in experimental rickettsial infection. *Mikrobiol Z* ; **58**:80-85.

Lucio-Villegas ME, Lozano de Leon F, Iglesias MC, Gómez-Mateos JM, López-Cortés L and Garcia-Bragado F. 1990. Malignant form of Mediterranean boutonneuse fever: importance of early treatment. *Enferm Infect Microbiol Clin*; **8**:394.

Maurin M and Raoult D. 1997. Bacteriostatic and bactericidal activity of levofloxacin against *Rickettsia rickettsii, Rickettsia conorii*, Israel spotted fever group rickettsia and *Coxiella burnetii*. *JAntimicrob Chemother*; **39**: 725–730.

Mumcuoglu KY and Keysary A. 2002. Mediterranean spotted fever in Israel: A tick-borne disease. *IMAJ*;**4**: 44-49.

Nafi O, Tarawnah Y and Tarawnah A**.** 2017. Epidemiological evaluation of Mediterranean spotted fever in children of the Karak province in south Jordan *Infect Dev Ctries*; **11**:242-246.

Paddock CD, Hilds JE, Sherif RZ and Rubinstein E**.** 1989. Mortality in Mediterranean spotted fever and murine typhus after treatment with chloramphenicol. *J Infect*;**18**: 35-37.

Parola P, Socolovschi C and Raoult D**.** 2009. Deciphering the relationships between *Rickettsia conorii* and *Rhipicephalus sanguineus* in the ecology and epidemiology of Mediterranean spotted fever. *Ann N Y Acad Sci* ; **1166**:49–54.

Purvis JJ and Edwards MS**.** 2000. Doxycycline use for rickettsial disease in pediatric patients. *Pediatr Infect Dis J*; **19**:871-874.

Raoult D, Gallais H, OttomaniA, Resch JP, Tichadou D *et al*. 1983. Malignant form of Mediterranean boutonneuse fever. 6 cases. *Presse Med*; **12**:2375–2378.

Raoult D and,Roux V. 1997. Rickettsioses as paradigms of new or emerging infectious diseases.. *ClinMicrobiol Rev*.;**10**: 694–719.

Raoult D, Weiller PJ, Chagnon A, Chaudet H, Gallais H and Casanova P.1986. Mediterranean spotted fever: clinical, laboratory and epidemiological features of 199 cases. *Am J Trop Med Hyg*; **35**:845-850.

Rovery C, Brouqui P and Raoult D. 2008. Questions on Mediterranean spotted fever a century after its discovery. *Emerg Infect Dis*; **14**:1360-1367.

Shaked Y, Samra Y, Maier MK and Rubinstein E. 1989. Relapse of rickettsial Mediterranean spotted fever and murine typhus after treatment with chloramphenicol. *J Infect*; **18**:35–37.

Stallings SP**.** 2001. Rocky Mountain spotted fever and pregnancy: a case report and review of the literature. *Obstet Gynecol Surv*; **56**:37–42.

Todd SR, Dahlgren FS, Traeger MS, Beltrán-Aguilar ED, Marianos DW, Hamilton C, McQuiston JH, Regan JJ. 2015**.** No visible dental staining in children treated with doxycycline for suspected Rocky Mountain spotted fever. *J Pediatr*. **166**:1246-51.

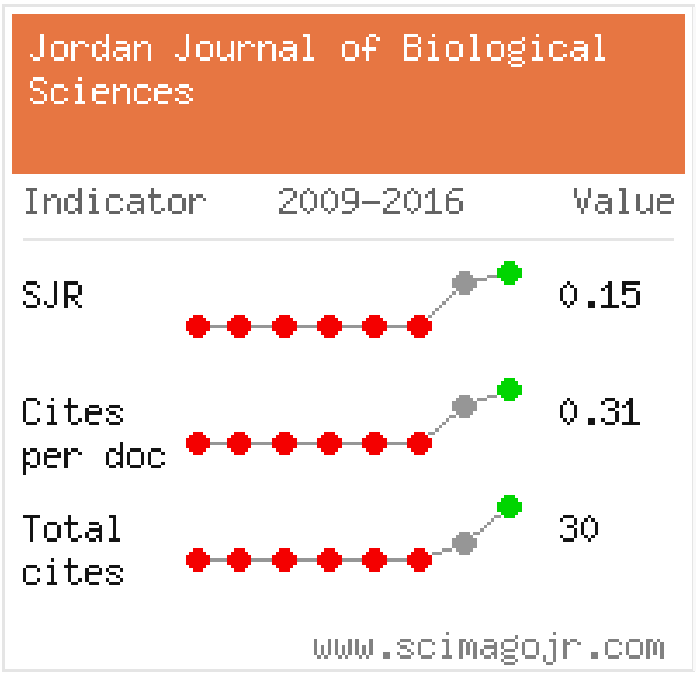
Wolach B, Franco S, Bogger-Goren S, Drucker M, Goldwasser RA *et al*. 1989. Clinical and laboratory findings of spotted fever in Israeli children. *Pediatr Infect Dis J*; **8**:152-155.

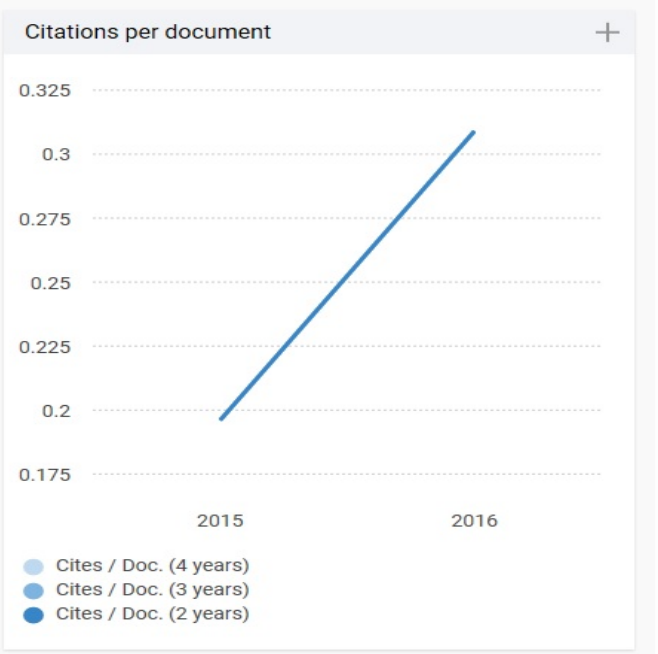
Weinberger M, Keysary A, Sandbank J, Zaidenstein R, Itzhaki A *et al*. 2008. Fatal *Rickettsia conorii* subsp. israelensis infection,Israel. *Emerg Infect Dis*;**14**: 821–882.

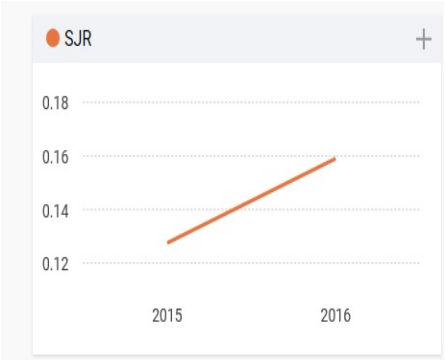
Woodward TE 1991**.** Rickettsial diseases. In: **Harrison's Principles of Internal Medicine**, 12th Ed , Wilson JD, Braunwald E, Isselbacher KJ, *et al* (Eds). 1991.. New York: McGraw-Hill;. pp. 489-490,756.

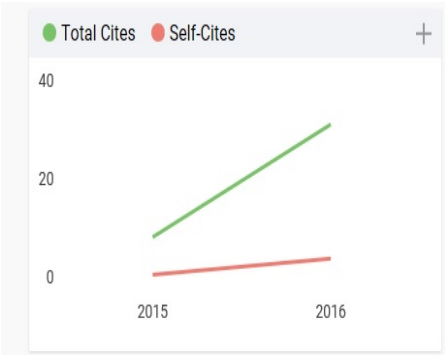
Yagupsky P, Gross EM, Alkan M and Bearman J. **1987.** Comparison of two dosage schedules of doxycycline in children with rickettsial spotted fever. *J Infect Dis*; **155**:1215-1219.

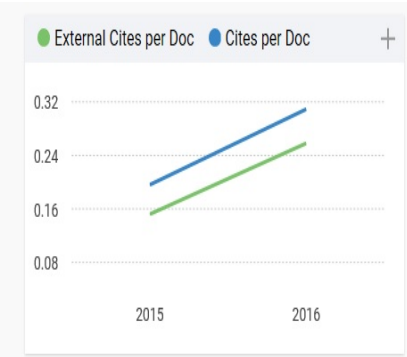
Appendix B

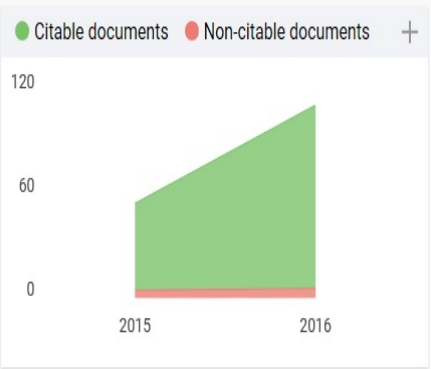


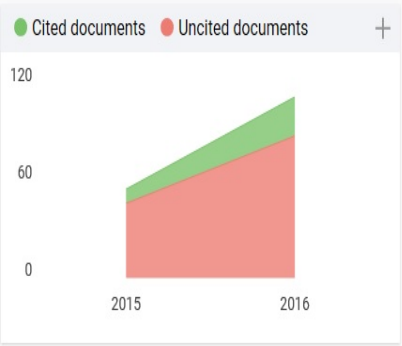












Title	Type	SJR	H index	Total Docs. (2016)	Total Docs. (3years)	Total Refs.	Total Cites (3years)	Citable Docs. (3years)	Cites / Doc. (2years)	Ref. / Doc.	
Jordan Journal of Biological Sciences	journal	0.146 Q4	3	30	102	974	30	97	0.31	32.47	

Subscription

Jordan Journal of
Biological Sciences

An International Peer – Reviewed Research Journal

Published by the deanship of Research & Graduate Studies, The Hashemite University, Zarqa, Jordan

Name: ..... الاسم:

Specialty: ..... التخصص:

Address: ..... العنوان:

P.O. Box: ..... صندوق البريد:

City &Postal Code:..... المدينة: الرمز البريدي:

Country: ..... الدولة:

Phone: ..... رقم الهاتف

Fax No.: ..... رقم الفاكس:

E-mail: ..... البريد الإلكتروني:

Method of payment: ..... طريقة الدفع:

Amount Enclosed: ..... المبلغ المرفق:

Signature: ..... التوقيع:

Cheques should be paid to Deanship of Research and Graduate Studies – The Hashemite University.

I would like to subscribe to the Journal

For

- ☐ One year
- ☐ Two years
- ☐ Three years

One Year Subscription Rates

	Inside Jordan	Outside Jordan
Individuals	JD10	$70
Students	JD5	$35
Institutions	JD 20	$90

Correspondence

Subscriptions and sales:

Prof. Khaled H. Abu-Elteen
The Hashemite University
P.O. Box 330127-Zarqa 13115 – Jordan
Telephone: 00 962 5 3903333 ext. 4357
Fax no. : 0096253903349
E. mail: jjbs@hu.edu.jo

المجلة الأردنية للعلوم الحياتية
Jordan Journal of Biological Sciences (JJBS)

http://jjbs.hu.edu.jo

المجلة الأردنية للعلوم الحياتية: مجلة علمية عالمية محكمة ومفهرسة ومصنفة، تصدر عن الجامعة الهاشمية وبدعم من صندوق البحث العلمي – وزراة التعليم العالي والبحث العلمي.

ترسل البحوث الى العنوان التالي:

رئيس تحرير المجلة الأردنية للعلوم الحياتية

الجامعة الهاشمية

ص.ب , 330127 , الزرقاء, 13115 , الأردن

هاتف: 0096253903333 فرعي 4357

E-mail: jjbs@hu.edu.jo, Website: www.jjbs.hu.edu.jo

المملكة الأردنية الهاشمية

المجلة الأردنية للعلوم الحياتية

مجلة علمية عالمية محكمة

تصدر بدعم من صندوق دعم البحث العلمي

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

ISSN 1995-6673