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 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.
Deanship of Scientific Research and Graduate Studies
The Hashemite University
P.O. Box 330127, Zarqa, 13115, Jordan
Phone: +962-5-3903333 ext. 4399
E-Mail: jjbs@hu.edu.jo

Hashemite Kingdom of Jordan

The Hashemite University

Jordan Journal of Biological Sciences

An International Peer-Reviewed Scientific Journal
Financed by the Scientific Research Support Fund

ISSN 1995-6673

Instructions to Authors

Scopes

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Manuscript, or the essence of their content, must be previously unpublished and should not be under simultaneous consideration by another journal.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	CABI
Geogle Scholar	EBSCO
Journal Seek	CAS (Chemical Abstract Service)
HINARI	ETH- Citations
Index Copernicus	Open J-Gat
NDL Japanese Periodicals Index	SCImago
SCIRUS	Science Citation Index (Zoological Abstract)
OAJSE	Scopus (in process)
ISC (Islamic World Science Citation Center)	Thomson Reuters (in process)
Directory of Research Journal Indexing (DRJI)	AGORA (United Nation's FAO database)
Ulrich's	SHERPA/RoMEO (UK)

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
Deanship of Scientific Research and Graduate Studies
Zarqa 13115 Jordan
Fax: +962 5 3903338
Email: jjbs@hu.edu.jo

Preface

Now commencing its seventh year, Jordan Journal of Biological Sciences (JJBS) will continue to provide biologists with first class research articles, review articles and short communications in various disciplines and frontiers of Biological Sciences. Here, I ask active researchers from all over the world to consider JJBS as one of their first choices for submission to publish their data. JJBS is now indexed with and included in DOAJ, EBSCO, CABI, HINARI, Google Scholar, Chemical Abstract Service, Zoological Abstract, Ulrich's, Index Copernicus International, ISC, Directory of Research Journal Indexing (DRJI) and others. Moreover, the journal is under the indexing process with ISI and Scopus. As always submitted research articles will receive fair and constructive comments by peer reviewers and worthwhile articles will get published. One of the great benefits we can provide to our prospective authors, regardless of acceptance of their manuscript or not, is the mentoring nature of our review process. JJBS provides authors with high quality, helpful notes and comments to assist authors in improving their manuscripts.

Moreover, the Editorial Board of JJBS are very much interested in publishing significant review articles that outline and discuss current hot topics in the frontiers of Biological Sciences. P utting such topics in perspective and fitting pertinent data together is of utmost importance in guiding future research and helping new scholars in the field to address important and pertinent issues. I encourage experts in various fields of Biological Sciences who wish to review certain front line topics in their specialties to contact me if they wish to contribute one or more review articles. In this way, the Editorial Board hopes to include at least one major or mini review in each journal issue as of March 2014.

As in prior two years, this seventh volume of JJBS will include four issues with at least twelve articles in each issue. In the coming year, it is my vision to have JJBS publishes more outstanding articles from distinguished scholars in various areas of Biological Sciences. In addition, I will be working on the inclusion of JJBS in Scopus, ISI and other international information retrieval services, which will lead to a good impact number.

Again, I must congratulate and thank all the researchers who contributed to research and review articles published in previous issues of JJBS during the past six years.

Also, I thank my esteemed reviewers of previous articles submitted to the journal. They are assurance of high quality of published research work. To all our former contributors and potential new ones, I welcome further manuscripts for submission. Your manuscripts will receive careful consideration to maintain a high quality publication in JJBS.

I would like to thank the JJBS International Advisory board members for their continuous support. Furthermore, I would like to thank the JJBS Editorial board members for their exceptional work and continuous support to JJBS. Finally, I very much appreciate the support of The Hashemite University and Jordanian Scientific Research Support Fund for their continuous support to JJBS.

Professor Khaled H. Abu-Elteen
Editor-in-Chief , JJBS
Hashemite University
Zarqa, Jordan
jjbs@hu.edu.jo

JJBS

Volume 7, Number3, September 2014
ISSN 1995-6673

Jordan Journal of Biological Sciences

CONTENTS

Original Articles

JJBS
Jordan Journal of Biological Sciences

Volume 7, Number 3, September .2014
ISSN 1995-6673
Pages 155 - 160

Pathogenicity of Syrian Isolates of *Bacillus thuringiensis* (Berliner) Against the Cereal Leafminer *Syringopais temperatella* Led. (Lep., Scythrididae) Under Laboratory Conditions

Basim A. Al-Dababseh[1]; Maysa A. Meihiar[2]; Firas A. Al-Zyoud[3] and Ihab H. Ghabeish[4,*]

[1]Department of Food Processing, Faculty of Agricultural Technology, Al-Balqa Applied University; [2] Plant Wealth Department , Ministry of Agriculture; [3] Department of Plant Protection and IPM, Faculty of Agriculture, Mu'tah University, Karak; [4]Department of Plant Production and Protection, Faculty of Agricultural Technology, Al-Balqa Applied University, Al-Salt 19117, Jordan.

Received: March 4, 2014 Revised: April 22, 2014 Accepted: May 6, 2014

Abstract

This study investigates the efficacy of 22 Syrian isolates of *Bacillus thuringiensis* (Berliner) (*Bt*) against *Syringopais temperatella* under laboratory conditions by feeding larvae on bacteria-contaminated barley leaves.The results indicated that all *Bt* isolates caused concentration and time related mortality, in which the highest mortalities were recorded at the highest concentration (original concentration), and at the latest time after *Bt* application(72 h).Mortality caused by all *Bt* isolates was significantly higher than mortality in the control. Isolate no. 1 gave significantly (P<0.05) the highest mortality percentage (73.3%) against the early larval instars. For the late larval instars, the isolates no. 21 and 22 were at bar with each other and gave significantly (P<0.05) the highest percentage of mortality (53.3%). Early larval instars were significantly (P<0.05) more susceptible to all concentrations of *Bt* isolates used than the late larval ones.In conclusion, the study showed that some of *B. thuringiensis* isolates tested in this study were pathogenic to *S. temperatella*.

Keywords: *Bacillus thuringiensis*, Cereal leafminer, Bio-insecticide, Biological control, Syrian *Bt* isolates, *Syringopais temperatella*.

1. Introduction

Wheat and barley are the main cereal crops grown in Jordan (Jordan Statistical Yearbook, 2011). One of the major constraints to the production of these crops in Jordan is the cereal leafminer, *Syringopais temperatella* Led. (Al-Zyoud *et al*., 2009). The pest causes serious annual forage and yield reduction. Outbreaks of this pest have mostly occurred in southern Jordan for the last 12 years (Al-Zyoud, 2013) with a crop infestation estimated at 25% to 75% (Al-Zyoud, 2012).

The use of synthetic insecticides is currently the predominant method of controlling *S. temperatella* in Jordan (Al-Zyoud, 2007; Al-Zyoud, 2008). In spite of the intensive use of insecticides to suppress the pest, the infested areas are continuously increasing(Al-Zyoud *et al*., 2011). In addition, the massive use of pesticides in modern agriculture caused their widespread diffusion to all environmental compartments including humans (Samiee *et al*., 2009). Increased public concerns about adverse effects of pesticides prompted search of alternative methods (i.e.,biopesticides) for pest control.

The most widely used biopesticide worldwide is the bacterium, *Bacillus thuringiensis* (Berliner) (*Bt*) (Lacey *et al*., 2001). *Bt* occurs naturally in soil, dead insects, water and grain dust (Bernhard *et al*., 1997; Schnepf *et al*., 1998). During the sporulation process, the bacteria produce large crystal proteins that are toxic to many insect pests (Daly and Buntin, 2005). When orally ingested by insects, the crystal δ-endotoxins proteins of *Bt* formed, which are highly toxic to insects (Candas *et al*., 2003; Balaraman, 2005; Roh *et al*., 2007).The *Bt* preparations are the most successful bio-control products worldwide (Kaur, 2002). *Bt* has been used to control lepidopteran, coleopteran and dipteran pests on food crops, ornamentals, forest trees and stored grains (Iriarte *et al*., 1998; Theunis and Aloali'i, 1999; Balaraman, 2005). Ammouneh *et al.* (2011) reported that *Bt* isolates are very toxic to *Ephestia kuhniella*, *Phthorimaea operculella* and *Cydia pomonella* larvae (Lepidoptera). In addition, Giustolin *et al.* (2001) and Gonzalez-Cabrera *et al.* (2011) found that *Bt* is highly efficient in controlling the tomato borer, *Tuta absoluta* (Meyrick). In spite of *Bt* was used in spray formulations for more than 40 years against nearly 3,000 species (Huang *et al*., 2004), only

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

one study has evaluated its efficacy on *S. temperatella* using the *israelensis* strain (Al-Zyoud *et al.*, 2011). However, since some *Bt* isolates produce diverse insecticidal proteins that show differential insecticidal activities (Meihiar *et al*., 2012), there is a high possibility to find some isolates which may be used to effectively control *S. temperatella*. Nevertheless, the continued damage to cereal crops by the pest is a daunting challenge and requires the use of new control methods. Therefore, this study aimed at evaluating the toxicity of 22 Syrian *Bt* isolates against *S. temperatella*. It is hoped that the application of *Bt* will play a central role in protecting cereal crops; thereby, it will drastically replace, at least in part, some of the most dangerous insecticides currently used against *S. temperatella*.

2. Materials and Methods

2.1. Rearing of Syringopais temperatella

The rearing of *S. temperatella* was initiated from hundreds of first larval instarscollected from a barley field in Al-Rabbeh/ Karak-Jordan (Latitude of 31°11" N and Longitude of 35°42" E, altitude of 980 m). The larvae were maintained on potted (12x12 cm) barley plants in a rearing room. The infested plants were kept in meshed cages of 50x50x50 cm under laboratory conditions of 20±5°C temperature, 60±10% relative humidity and 12: 12 (L: D) h photoperiod. Barley of Mu'tah cultivar was used for rearing the pest and conducting the experiments. The plants were frequently replaced whenever needed to maintain adequate host-plant supply. Two groups of larvae were used in the experiments, which were randomly selected from the culture. The first group consisted of early larval instars ($L_1$and L_2), while the second group consisted of late larval instars (L_4 and L_5).

2.2. Culturing, Preparation and Characterization of Bacillus thuringiensis Isolates

The *Bt* isolates were obtained from Dr. Maysa Meihiar as 22 biochemical types. *Bt* was isolated from Syrian soils of different plant communities (Meihiar *et al.*, 2012) following the method described by Ohba and Aizawa (1986). *Bt* subsp. *Kurstaki* HD1 (Abbott Laboratories, Chicago, USA) was used as a reference for comparison with the previous results of the nearest insect species (*Ephestia kuehniella* Zeller) and also as a positive control for results of the present study. One ml of each *Bt* isolate-suspension was added into 10 ml of T_3 medium (g per liter: 3 tryptone, 2 tryptose, 1.5 yeast extract, 0.05 M sodium phosphate at pH 6.8 and 0.005 of $MnCl_2$). The suspension was incubated at 30ºC for 7 days until the bacterial cells have sporulated for crystal production (Travers *et al*., 1987; Martin and Travers, 1989). In order to know the number of viable cells at the time of bioassay and after 7 days of incubation,1 ml of each of the 22 cultures was separately poured in 9 ml of sterile distilled water, and hereafter 9-fold serial dilutions were made. Aliquots of 1 ml of 7-9 fold dilutions were plated in duplicate by pour-plate technique using nutrient agar, and incubated at 30ºC for 2 days. The colony forming units (CFU) were visually counted for the 22 different bio-assayed isolates (Kango, 2010) (Table 1).

Table 1. Colony forming units (CFU) and shape of crystals of the twenty two bio-assayed *Bacillus thuringiensis* soil isolates.

Isolate no.	Isolate subsp.	CFU/ml (mean)	Crystal morphology
Bt 1	Untypable	9.00 E + 08	Bipyramidal + Cuboidal
Bt 2	Untypable	3.50 E + 09	Bipyramidal + Cuboidal
***Bt*3**	***Kurstaki* HD1**	**6.00 E + 09**	**Bipyramidal + Cuboidal**
Bt 4	*kurstaki*	5.20 E + 09	Bipyramidal + Cuboidal
Bt 5	Untypable	8.25 E + 09	Spherical + Bipyramidal + Cuboidal
Bt 6	*Sotto*	1.50 E + 11	Bipyramidal
Bt 7	Untypable	1.44 E + 11	Rectangular + Bipyramidal + Cuboidal
Bt 8	Untypable	1.05 E + 11	Irregular + Bipyramidal + Cuboidal
Bt 9	Untypable	1.57 E + 11	Bipyramidal + Cuboidal
Bt 10	Untypable	1.95 E + 09	Bipyramidal + Cuboidal
Bt 11	Untypable	9.50 E + 08	Rectangular + Bipyramidal + Cuboidal
Bt 12	*kurstaki*	2.50 E + 08	Bipyramidal + Cuboidal
Bt 13	*kurstaki*	8.50 E + 08	Bipyramidal + Cuboidal
Bt 14	Untypable	1.65 E + 10	Rectangular + Bipyramidal + Cuboidal
Bt 15	Untypable	8.50 E + 09	Spherical + Bipyramidal + Cuboidal
Bt 16	Untypable	9.00 E + 08	Rectangular + Bipyramidal + Cuboidal
Bt 17	Untypable	2.15 E + 09	Spherical + Bipyramidal + Cuboidal
Bt 18	Untypable	9.50 E + 08	Rectangular + Bipyramidal + Cuboidal
Bt 19	Untypable	1.45 E + 09	Rectangular + Bipyramidal + Cuboidal
Bt 20	Untypable	1.60 E + 10	Rectangular + Bipyramidal + Cuboidal
Bt 21	Untypable	1.00 E + 09	Like*Bt*subsp. *Finitimus* (rhomboid in shape)
Bt 22	Untypable	1.00 E + 09	Bipyramidal + Cuboidal

**Bt* subsp. *Kurstaki* HD1 (Dipel®- Abbott Laboratories, Chicago, USA) used as a reference strain

2.3. Bioassay

The bioassay was set up in Petri-dishes of 5.5 cm in diameter that were partially filled with 0.5 cm thick layer of wetted cotton pad. The lid of each Petri-dish had a hole closed with organdie fabric for ventilation. Barley leaf strips of10 cm^2area, which were cut from un-infested barley plants, were placed in the Petri-dishes. Either *S. temperatella* early or late larval instars were gently transferred using a Camel hairbrush into the Petri-dishes in groups of ten larvae/Petri-dish. Larvae in the control treatments (n=10) were provided with leaf strips dipped in sterilized distilled water for 30 sec, while larvae in the *Bt* treatments (n=10) were provided with leaf strips dipped in an aqueous solution of the required *Bt* isolate and concentration (original and twofold serial dilutions) for 30 sec. The Petri-dishes were kept under the above-mentioned laboratory conditions. Larval mortality was recorded 1, 2 and 3 days post-treatment with *Bt*. Larvae were considered dead if they do not move when gently prodded with fine brush. Data obtained for mortality percentages of early and late larval instars of *S.*

temperatella were plotted as a linear correlation function versus the CFU.

2.4. Statistical Analysis

The statistical analysis was performed using the proc GLM of the statistical package Sigma Stat version 16.0 (SPSS, 1997). The experimental design was a complete randomized design (CRD) with three replications for each isolate and concentration. The data were analyzed using one way ANOVA to detect any differences in the pest mortality (Zar, 1999). When significant differences were detected, means were compared using LSD at 0.05 probability level (Abacus Concepts, 1991). The *t-test* was used for comparison between only two means (Anonymous, 1996). In addition, the correlation between mortality and progress of time after spraying as well as mortality and concentration of *Bt* was calculated by Spearman's correlation method (Zar, 1999).

3. Results

The results presented herein are for *Bt* isolates as an original concentration (named conc. 1), first-fold dilution (named conc. 2) and second-fold dilution (named conc. 3). However, the results revealed that the original concentration of *Bt* (ranged between CFU = 2.5×10^{8} for isolate *Bt* 12 (*Bt kurstaki*) to 1.57×10^{11} for isolate *Bt* 9) gave the highest percentage of mortality for both *S. temperatella* early (F=4.250; 2, 195 df; P=0.016) and late (F=6.655; 2, 195 df; P=0.002) larval instars tested, but it was at bar with the first-fold dilution, while it differed significantly from the second-fold dilution (Figure 1). The mortality percentages resulted from the original and the second dilutions were 20.8% and 12.5% (early instars), and 16.5% and 8.6% (late instars), respectively. Furthermore, early larval instars were significantly more susceptible to all *Bt* isolates and concentrations used than the late larval instars (Figure 1).

The highest larval mortality was recoded after 72 hr of *Bt* application, and it was significantly different from those at24 and 48 hr post application for both early (F=6.990; 2, 63 df; P=0.002) and late larval instars (F=22.987; 2, 63 df; P=0.000) (Figure 2). Mortalities of 26.8% and 21.0% for early and late larval instars' were reported 72 hr post *Bt* application, respectively, while the mortalities were only 9.6% and 3.6% after 24 hr of *Bt* application, respectively. Early larval instars were more susceptible to *Bt* than the late instars at all times after application (24, 48 and 72 hr) (Figure 2).

Overall mortality percentage of *S. temperatella* as a result of leaf-dipping in the three different concentrations of *Bt* isolates for both early and late larval instars are presented in Figure 3. Mortality caused by all *Bt* isolates was significantly higher than that of the control. Moreover, the isolates; *Bt*1, *Bt* 21 and *Bt*13 (*Bt kurstaki*) caused significantly the highest mortality percentages with 28.2%, 26.5% and 25.7%, respectively (F=4.884; 22, 391 df; P=0.000). In contrast, the isolates; *Bt*8, *Bt*17 and *Bt*4 (*Bt kurstaki*) caused significantly the lowest mortality with 4.3%, 5.8% and 5.9%, respectively.

Since the results in Figure 1 and 2 showed that the highest efficacy of *Bt* was obtained 72 hr post *Bt* application at the original concentration, further statistical analysis was made at these time and concentration to check the efficacy of all *Bt* isolates tested as shown in Figure 4. The results indicated, for early larval instars, that the isolate *Bt* 1 gave significantly the highest mortality percentage (73.3%), followed by the isolates; *Bt* 13 (*Bt kurstaki*) (66.7%), 19 (63.3%) and 16 (63.3%), while the isolates; *Bt* 8, *Bt*9, *Bt* 17, *Bt*14 and *Bt* 12 (*Bt kurstaki*) were at bar with the control treatment (mortality: 0.0-6.7%) (F=23.040; 22, 46 df; P=0.000). For late larval instars, the isolates; *Bt* 21 and *Bt*22 were at bar with each other and gave significantly the highest percentage of mortality (53.3%). Moreover, all isolates gave significantly higher mortalities to the late larval instars than control (F=20.996; 22, 46 df; P=0.000). Out of 22 isolates, three isolates (*Bt*20, *Bt*21 and *Bt*22) caused higher mortality for both early and late larval instars.

There was a positive significant correlation between time after *Bt* application and mortality percentage as well as between *Bt* isolate's concentration and mortality percentage (Table 2). This means that with increasing time after *Bt* application and *Bt* concentration there was an increase in the mortality of *S. temperatella*. No clear relationship was noticed between the efficacy of *Bt* isolates and shape of crystal proteins they have; however, it is to be mentioned that most of the effective and ineffective isolates have bipyramidal and cuboidal parasporal crystal protein shapes. The correlation between CFU and larval mortality was plotted for the promising isolates (that gave mortality higher than 40%) against early (Figure 5A) and late (Figure 5B) larval instars. The results indicated that the relationship isof increasing function; higher mortalities were obtained from effective isolates of higher CFU.

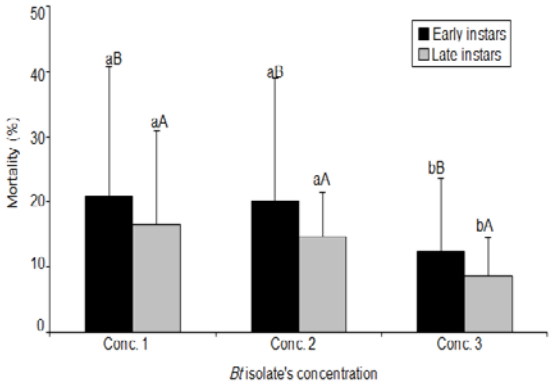

Figure 1. Mortality percentage (±SD) of *Syringopais temperatella* in relation to larval instars resulted from application of different *Bacillus thuringiensis* isolates in a feeding larval test on bacterial contaminated barley leaves under laboratory conditions. [Different small letters above bars indicate significant differences among the different *Bt* concentrations within the same instar, while capital letters above bars indicate significant differences between the different instars within the same concentration at p<0.05 (one-factor analysis of variance)].

Table 2. Correlation analysis of mortality percentage of *Syringopais temperatella* versus time and concentration of *Bt* isolates in a feeding larval test on bacteria-contaminated barley leaves under laboratory conditions.

Correlated variables	Instar	R value	Significance
Time vs. Mortality percentage	Early instars	0.411**	0.001
	Late instars	0.707**	0.000
	Both (early and late)	0.682**	0.000
Bt isolate's concentration vs. Mortality Percentage	Early instars	0.233*	0.050
	Late instars	0.385**	0.001
	Both (early and late)	0.365**	0.003

*Correlation is significant at the 0.05 probability level.
**Correlation is significant at the 0.01 probability level

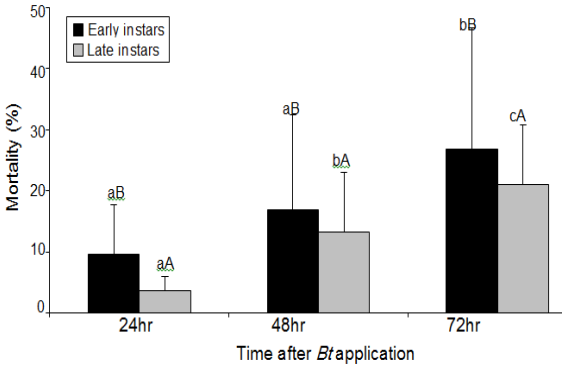

Figure 2. Mortality percentage (±SD) of *Syringopais temperatella* in relation to time after *Bt* application resulted from different isolates in a feeding larval test on bacterial contaminated barley leaves under laboratory conditions. [Different small letters above bars indicate significant differences among the different times within the same instar, while capital letters above bars indicate significant differences between the different instars within the same time at $p<0.05$ (one-factor analysis of variance)].

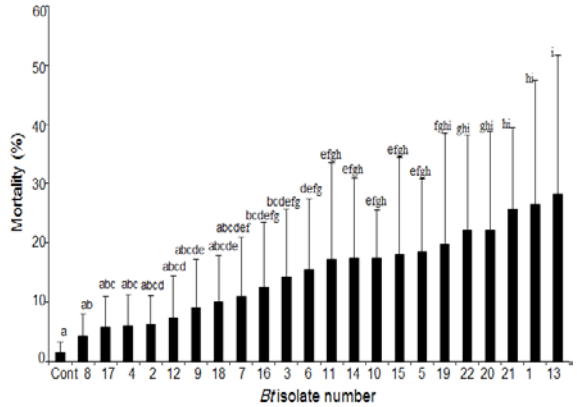

Figure 3. Overall effect of *Bacillus thuringiensis* different isolates (all concentrations) on *Syringopais temperatella* mortality percentage (±SD) in a feeding larval test on bacterial contaminated barley leaves under laboratory conditions.Isolate no. 3 (*Bt* subsp. *kurstaki* HD1) is the reference strain.[Different small letters above bars indicate significant differences among the different *Bt* isolates at $p<0.05$ (one-factor analysis of variance)].

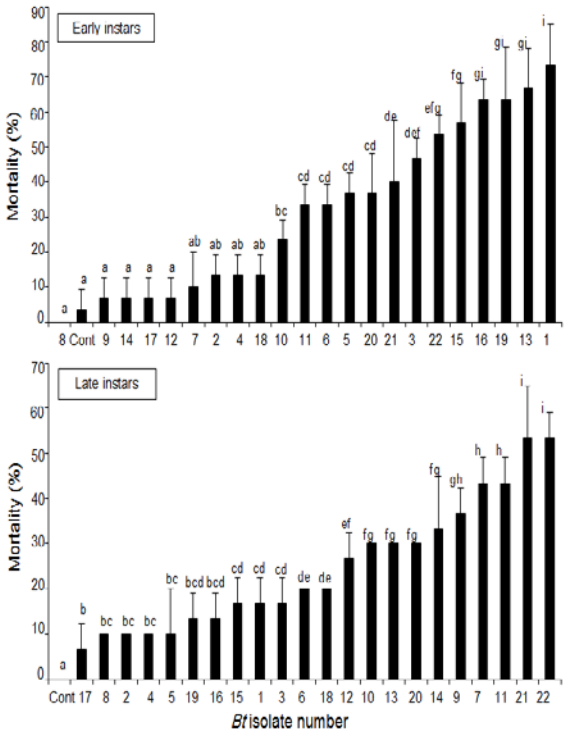

Figure 4. Mortality percentage (±SD) of *Syringopais temperatella* early and late larval instars as a result of application by the higher concentration of *Bacillus thuringiensis* different isolates after 72 hrin a feeding larval test on bacterial contaminated barley leaves under laboratory conditions.Isolate no. 3 (*Bt* subsp. *kurstaki* HD1) is the reference strain.[Different small letters above bars indicate significant differences among the different *Bt* isolates at $p<0.05$ (one-factor analysis of variance)].

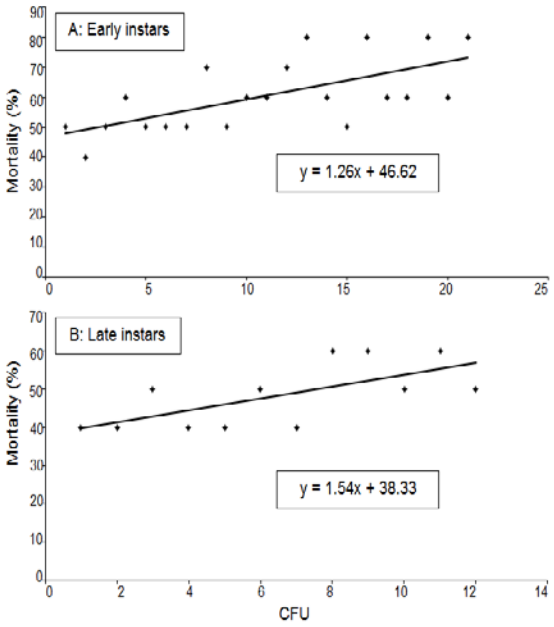

Figure 5. Scatter diagram with a line of best fit curve of obtained mortality percentages of the early (A) and late (B) larval instars of *Syringopais temperatella* after 72 hr as a function of colony forming units (CFU) of the effective *Bacillus thuringiensis* isolates.

4. Discussion

In this study four variables were considered; the *Bt* isolates (22 different isolates), the target larval instar (early and late), the *Bt* concentration (original, first-fold and second-fold dilutions) and the effect of time after exposure to *Bt* (24, 48 and 72 hr). The reference strain we used (*Bt* subsp. *kurstaki* HD1) is the same used by Meihiar *et al.* (2012). We reported 48% mortality for the reference strain against *S. temperatella* while 53% mortality was reported by Meihiar *et al.* (2012) against *E. kuehniella* from the same insect order. For the 72 hr-mortality at the higher *Bt* concentration used, 6 and 2 isolates out of the 22 isolates were effective against early and late larval instars of *S. temperatella*, respectively. They reported mortality percentages more than that reported for the reference strain. We think that these promising isolates produce *cry* gene toxic against the pest larvae as reported by many researchers (Tabashnik *et al.*, 1994; Porcar *et al.*, 2000; Song *et al.*, 2003). In addition, the level of gene expression plays a role in the toxicity as proposed by Porcar *et al.* (2000). However, the *Bt* toxicity did not depend on *cry* gene content only, because factors other than *cry* proteins may contribute to the toxicity as well as spore interaction with crystal protein and the other soluble toxins such as β–exotoxin (Porcar *et al.*, 2000). Moreover, Martinez *et al.* (2004) suggested that the biological activity of a strain cannot be fully predicted on the basis of its *cry* gene content alone. The relative proportion of the *cry* proteins produced, their interaction, and the possible presence of undetected crystal proteins are all important. The present results revealed that the isolate number 13 (*Bt kurstaki*) caused 68% mortality after 3 days of exposure to early instar larvae of *S. temperatella* which is coincide with the previously reported results for this bacteria subspecies using the same pest with 71.4%% mortality (Al-Zyoud *et al*., 2011). Furthermore, we suggest that isolates are mostly produced the toxic proteins after two days of infection. What Al-Zyoud *et al.* (2011) found is supporting our suggestion, where they found that a gradual increase in mortality with time post *Bt* spraying against *S. temperatella* larvae. Moreover, the current results indicated that all *Bt* isolates caused concentration related mortality, in which the highest mortality was recorded at the highest concentration. Therefore, the results showed that both time and concentration play an important role in the bacteria efficacy. On the contrary of Obeidat *et al.* (2004) and Meihiar *et al.* (2012) findings, the shape of the isolates' crystal proteins did not correlate with the isolate efficacy; where bipyramids and cuboids crystal proteins were found in our both effective and ineffective isolates. Obeidat *et al.* (2004) found that out of the twenty-six strains of *Bt*, serotypes *kenyae*, *kurstaki* and *kurstaki* HD1 produced bipyramid crystal proteins which were toxic to *E. kuehniella*. Moreover, Meihiar *et al.* (2012) confirmed Obeidat *et al.*(2004) finding, in which *Bt* strains producing bipyramid and cuboid crystal shapes are the most toxic to the same insect. In the current study, the remaining 11 isolates were found less effective against the *S. temperatella* larvae. The reason behind this is that such isolates might not produce *Cry* proteins or their genes were of low level of expression. In addition, the same isolates investigated in this study were previously bio-assayed against *E. kuehniella* by Meihiar *et al.*(2012), and PCR was used to examine their *cry* genes content. Their findings demonstrated that the most toxic isolates harbor different specific *cry* genes including *cryI* and *cryIV* which have an insecticidal activity to lepidopteran insects. This finding is also proposed previously by Ammouneh *et al*. (2011), in which the type of the *cry* genes was found to correlate with its insecticidal activity.

The present results revealed that the isolate *Bt* 1 was effective in controlling the early instars larvae but it showed low efficacy against late instars. This might be due to the low number of bacterium cells in the original concentration due to the low sporulation rate, and as a result low number of crystal proteins (as shown in Table 1)as compared with the isolates; *Bt* 21 and *Bt* 22, which were efficient against the late instars. Therefore, this little number of crystal proteins in the isolate could not produce enough amounts of toxins to affect the late larval instars which are much larger in size than the early ones. This justification is supported in our findings (as shown in Figure 5) as indicated by the relationship between the number of bacterial cells and the mortality obtained. This relationship is of a positive trend; the more number of bacterial cells the higher the mortality obtained. It is to be mentioned that the number of bacterial cells is positively correlated with the amount of crystals and subsequently the amount of toxins they produced to cause death to the pest larvae.

In conclusion, some of *Bt* isolates used in this study exhibit a toxic potential against the pest and, therefore, they could be adopted for future control program to suppress the pest as a part of IPM program, and thus will reduce and/or replace the most dangerous chemical insecticides currently used against the pest in Jordan and surrounding countries. Better pest control strategy can increase farm incomes and reduce the hazards in rural areas associated with insecticides' use as well as will contribute to improve food security where wheat and barley are major sources of food for human and their animals in the region.

References

Abacus Concepts. 1991. **SuperAnova user's manual**. Version 1.11, Berkeley, CA.

Al-Zyoud F. 2007. Investigations on certain biological and ecological parameters of the cereal leafminer *Syringopias temperatella*Led. (Lep., Scythrididae). *Bull Fac Agric, Cairo Univ*. **58**: 164-172.

Al-Zyoud F. 2008.Effects of direct spray and residual exposure of different insecticides on the cereal leafminer *Syringopais temperatella*Led. (Lep., Scythrididae) under laboratory conditions. *Jordan J Agric Sci*.,**4**: 1-11.

Al-Zyoud F. 2012. Effect of field history on the cereal leafminer *Syringopais temperatella* Led. (Lepidoptera: Scythrididae) and its preference to different wheat and barley cultivars. *Pakistan J Biol Sci*., **15**: 177-185.

Al-Zyoud F. 2013. Efficacy of insecticides' applications against the cereal leafminer *Syringopais temperatella*Led. (Lep., Scythrididae) on barley under field conditions in Karak-Jordan. *Dirasat, Agri. Sci*.**,39**: 65-74.

Al-Zyoud F, Al-Emiri N and Al-Omari A. 2011. Efficacy of the insecticidal bacterium *Bacillus thuringiensis* (Berliner) (*Bt*) against the cereal leafminer *Syringopais temperatella* Led. (Lep., Scythrididae) under laboratory conditions. *Jordan J Agric Sci*.,7: 83-92.

Al-Zyoud F, Salameh N,Ghabeish I and Saleh A. 2009. Susceptibility of different cultivars of wheat and barley to cereal leafminer *Syringopais temperatella* Led. (Lep., Scythrididae) under laboratory conditions. *J Food Agric Environ*.,**6**: 235-238.

Ammouneh H,Harba M,Idris E and Makee H. 2011. Isolation and characterization of native *Bacillus thuringiensis* isolates from Syrian soil and testing of their insecticidal activities against some insect pests. *Turk J Agric Forest*., **35**: 421-431.

Anonymous.1996. **Reference Manual of the Statistics Program for Windows Winstat**. Kalmia Company Inc, Cambridge, MA, p. 267.

Balaraman K. 2005. Occurrence and diversity of mosquitocidal strains of *Bacillus thuringiensis*. *J Vect Borne Dis*.,**42**: 81-86.

Bernhard K, Jarret P, Meadows M, Butt J, Ellism DJ, Roberts GM, Pauli S, Rodgers P and Burges HD. 1997. Natural isolates of *Bacillus thuringiensis*: Worldwide distribution, characterization, and activity against insect pests. *Appl Environ Microbiol*.,**70**: 59-68.

Candas M,Loseva O,Oppert B,Kosaraju P and Jr. Bulla LA. 2003. Insect resistance to *Bacillus thuringiensis*: alterations in the Indian meal moth larval gut proteome. *Molec Cell Proteomics*, **2**: 19-28.

Daly T and Buntin GD.2005. Effect of *Bacillus thuringiensis* transgenic corn for Lepidopteran control on non target arthropods. *Environ Entomol*.,**34**: 1292-1301.

Giustolin TA,Vendramim JD, Alves SB, Vieira SA and Pereira RM. 2001. Susceptibility of *Tuta absoluta* (Meyrick) (Lep., Gelechiidae) reared on two species of Lycopersicon to *Bacillus thuringiensis* subsp. *kurstaki*. *J Appl Entomol*.**, 125**: 551-556.

Gonzalez-Cabrera J, Molla O, Monton H and Urbaneja A.2011. Efficacy of *Bacillus thuringiensis* (Berliner) in controlling the tomato borer *Tuta absoluta* (Meyrick) (Lep., Gelechiidae). *BioControl***, 56**: 71-80.

Huang Z, Guan C and Guan X. 2004. Cloning, characterization and expression of a new *cry1* Ab gene from *Bacillus thuringiensis* WB9. *Biotech. Lett*., **26**: 1557-1561.

Iriarte J, Bel Y,Ferrandis MD, Andrew R, Murillo JF and Caballero P.1998. Environmental distribution and diversity of *Bacillus thuringiensis*in Spain. *Syst Appl Microbiol*.**, 21**: 97-106.

Jordan Statistical Yearbook. 2011. Department of Statistics/**Annual Agriculture Surveys**. Published online:http://www.dos.gov.jo/dos_home_a/main/cd_yb2011 /pdf/agri.pdf.

Kango N. 2010. **Textbook of Microbiology**. I. K. International Publishing House Pvt. Ltd., India.

Kaur S. 2002. Potential for developing novel *Bacillus thuringiensis*strains and transgenic crops and their implications for Indian agriculture. *Ag Biotech Net***, 4**: 1-10.

Lacey LA,Frutos R, Kaya HK and Vail P.2001. Insect pathogens as biological control agents: Do they have a future? *Biol. Cont.*, **21**: 230-248.

Martin Pand Travers R.1989. Worldwide abundance and distribution of *Bacillus thuringiensis* isolates. *Appl Environ Microbiol*., **55**: 2437-2442.

Martinez C, Porcar M, Lopez A, de Escudero FJ, Perez-Llarena IR and Caballero P. 2004. Characterization of a *Bacillus thuringiensis* strain with a broad spectrum of activity against Lepidoptera insect. *Entomol Exp. Appl*., **111:** 71-77.

Meihiar M, Ahmad M and Kebebo E.2012. Isolation and characterization of different *Bacillus thuringiensis* strains from Syria and their toxicity to the Mediterranean flour moth *Ephestiakuehniella* Zeller (Lep., Pyralidae). *Jordan J Agric Sci.*, **8**: 196-207.

Obeidat M,Hassawi D and Ghabeish I.2004. Characterization of *Bacillus thuringiensis* strains from Jordan and their toxicity to the Lepidoptera, *Ephestiakuehniella* Zeller. *African J Biotech.*, **3**: 622-626.

Ohba M and Aizawa K.1986. Distribution of *Bacillus thuringiensis* in soils of Japan. *J Inverteb Pathol*., **47**: 277-282.

Porcar M, Martinez C and Caballero P. 2000. Host range and gene contents of *Bacillus thuringiensis* strains toxic towards *Spodopteraexigua*. *Entomol Exp Appl*., **97**: 339-346.

Roh JY, Jae YC, Ming SL,Byung RJ and Yeon HE. 2007. *Bacillus thuringiensis*as a specific, safe, and effective tool for insect pest control. *J Microbiol Biotechnol*., **17**: 547-559.

Samiee A,RezvanfarA and Faham E. 2009. Factors influencing the adoption of integrated pest management (IPM) by wheat growers in Varamin County, Iran. *African J Agric Res*., **4:** 491-497.

Schnepf E,Crickmore N, Van Rie J,Lereclus D, Baum J,Feitelson J, Ziegler DR and Dean DH.1998. *Bacillus thuringiensis*and its pesticidal proteins. *Microbiol Molec Biol Rev*., **62**: 775-806.

Song F, Zhang J, Gu A, Wu Y, Han L, He K, Chen Z, Yao J, Hu Y, Li G and Huang D. 2003. Identification of *cry*1I-type genes from *Bacillus thuringiensis* strains and characterization of a novel *cry*1I-type gene. *Appl Envir Microbiol*., **69**: 5207-5211.

SPSS, Statistical Product and Service Solutions INC. 1997. **SIGMASTAT 2.03: SigmaStat Statistical Software User's Manual**. Chicago, United States.

Tabashnik BE, Finson N, Johnson MW and Heckel DG. 1994. Cross-resistance to *Bacillus thuringiensis*toxin Cry1F in the Diamondback moth (*Plutellaxylostella*). *Appl Environ Microbiol.*, **60**: 4627-4629.

Theunis W and Aloali'I I. 1999. Susceptibility of the Taro beetle, *Papuanauninodis* (Coleoptera:Scarabaeidae) to two new *Bacillus popilliae* isolates from *Papuana* spp. *J Invert Pathol*., **73**: 255-259.

Travers R,Martin P and Reichelderfer C.1987. Selective process for efficient isolation of soils *Bacillus* spp. *Appl Environ Microbiol*., **53**: 1263-1262.

Zar J. 1999. **Bio-statistical analysis**. Prentice Hall, Upper Saddle River, NJ, pp 663.

JJBS

Volume 7, Number 3, September .2014
ISSN 1995-6673
Pages 161 - 164

Jordan Journal of Biological Sciences

Inhibitory Effect of Mediterranean Sage and Rosemary on Clinical and Community Isolates of Methicillin-Resistant *Staphylococcus aureus*

Hamed M. Alzoubi[1,*], Abdallah I. Ibrahim[2], Mohammed S. Alsbou[3] and Amin A. Aqel[1]

[1]*Microbiology and Immunology Department, Faculty of Medicine, Mu'tah University;*

[2]*Medical Laboratory Sciences, Faculty of Allied Medical Sciences, Tabuk University, Tabuk 741, Saudi Arabia;*

[3]*Pharmacology Department, Faculty of Medicine, Mu'tah University, Alkarak 61710, Jordan.*

Received: April 17, 2014 Revised: May 6, 2014 Accepted: May 9, 2014

Abstract

Plant extracts are traditionally used for treating many infectious and non- infectious diseases. This study aimed at assessing the inhibitory effect of the ethanol extracts of two medicinal plants; Mediterranean Sage (*Salvia libanotica*) and Rosemary (*Rosmarinus officinalis*) on clinical and community strains of methicillin-resistant *Staphylococcus aureus* (MRSA). Ethanol extracts of the two plants were tested for their antibacterial effect against 25 clinical (n=15, 60%) and community (n=10, 40%) strains of MRSA. Rosemary and Mediterranean Sage extracts demonstrated activity against all isolates, 50µl of 100 mg/ml of each plant extract yielded inhibition zone reaching as high as 27 and 30 mm by agar well diffusion method. Effective MICs and MBCs of ethanol extracts of Rosemary and Mediterranean Sage against MRSA were 0.125 to 0.5mg/ml and0.25 to 1 mg/ml respectively. Mixed ethanol extract of Rosemary and Mediterranean Sage showed antagonistic effect on MRSA strains. These results suggest the potential therapeutic implications of the ethanol extract from Rosemary and Mediterranean sage in the treatment of MRSA infections.

Key words: Mediterranean Sage; Rosemary; MRSA; Jordan.

1. Introduction

Traditionally, the dependence on alternative medicine in developing countries and other parts of the world is clearly obvious, they use preparations of any part of the plants for the purpose of pain relief, infection prevention or even as cosmetics (Ahmad *et al.,* 1998). Studies evaluating medicinal plants as a source of antimicrobials proved that plants' active components might be used as bacterial inhibition agents (Emori and Gaynes, 1993; Cos *et al*., 2006). Medicinal plants may play a role as a natural source of antimicrobial drugs (Habeeb *et al.,* 2007).

Methicillin-resistant *Staphylococcus aureus* (MRSA) infections of both hospital and community acquired have increased remarkably during the last decade (Chambers, 1997).Hospital acquired MRSA (HA-MRSA) strains are mainly distinguished from community acquired MRSA (CA-MRSA) using molecular techniques. The S*CC mec* in HA-MRSA belongs to type I, II or III and is bigger than that of CA-MRSA, which belongs to S*CC mec* type IV or V. In addition, CA-MRSA strains frequently carry the gene for PVL (Panton Valentine Leukociden) toxin which is not commonly found in HA-MRSA (Naimi *et al.,* 2003). Antibiotic options for patients with MRSA infection are usually restricted due to the wide range of MRSA antibiotic resistance. This has enhanced researchers to use other natural agents to fight MRSA, especially from medicinal plants (Schito, 2006).In Jordan, data revealed that MRSA infections both hospital and community acquired have been increased in the last few years, with percentage of 62% and 8% respectively (Borg *et al.,* 2007; Aqel *et al.,* 2012).

In a previous study carried out by some authors of this study (Ibrahim *et al.*,2013), the effect of crud extract of *S. libanotica* and *R. officinalis* against two test strains of *Staphylococcus aureus* ATCC (25923) and an MRSA isolate was clearly identified. The present study aims to further assess the antimicrobial effect of *S. libanotica* and *R. officinalis* against clinical and community isolates of MRSA. Both Mediterranean Sage and Rosemary tested in this study are used traditionally in Jordan for purposes such as the treatment or relief of respiratory and gastrointestinal infections (Obeidat, 2011; Abu-Shanab *et al.*, 2004).

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

2. Materials and Methods

2.1. Plant Samples and Manipulation

Two medicinal plants, *Salvia libanotica* (Mediterranean Sage) and *Rosmarinus officinalis* (Rosemary) were tested in this study (Table 1).The experimental part of the study was done in the department of microbiology and immunology, faculty of medicine, Mu'tah University, from 1 May 2013 to 15 September 2013. Plants were purchased locally from the markets. Leaves were collected to be air dried for 14 days and then powdered using mortar and pestle**.**

Table 1. Profile of the two medicinal plants used

Botanical Name	Family	Local Name	Plant part used
Rosmarinus officinalis	Lamiaceae	Hasa-alban	leaves
Salvia libanotica	Lamiaceae	Miramiyah	leaves

2.2. Ethanol Extracts Preparation

One hundred grams of each powder were extracted by cold maceration with 80% ethanol for 48 hrs at room temperature. Filtration by Whatman filters paper no. 2; evaporation and concentration of the extract under low pressure were applied consequently for each plant extract (Ahmad *et al.,* 1998).Powder samples were stored at 4°C. Primary active components and essential oils extraction were performed according to Böszörményi (2009). Briefly, oil extraction was done under water steam distillation for 3 hrs of 30 gm of the plants powder.

2.3. Test Organisms

A total of 25 non-repeat MRSA strains, 15 (60%) strains from different patients admitted to Al-Karak hospital, Jordan, and other 10 (40%) MRSA nasal swabs strains obtained from healthy individuals in the year 2013 were studied. The clinical strains were isolated from respiratory samples (n = 3; 20%), wound swabs (n = 3; 20%), urine (n =4; 26.6%), pus (n = 1; 6.7%), catheter tip (n = 1; 6.7%), blood (n = 3; 20%).Isolates were identified morphologically and biochemically by standard microbiological procedures using Gram stain, catalase test, coagulase test and an API system (bioMérieux, France). Cefoxitin (30 µg) discs (Oxoid, Basingstoke, UK) were used for methicillin resistance determination. Susceptibility tests were applied according to guidelines of Clinical Standards Laboratory Institute (CLSI, 2012). Detection of *MecA* gene (encoding high-level resistance to methicillin) and16S rRNA gene (internal control) were performed with DNA extraction, primers and amplification conditions according to Petinaki (2001). *Staphylococcus aureus* ATCC 29213 and 2 methicillin-susceptible *Staphylococcus aureus* **(**MSSA) strains were used as control strains during susceptibility testing and PCR procedures. Bacterial samples were preserved in transport media at 4°C and subcultured overnight before use. Consent was obtained from all participants after explaining the purpose of the study. The ethics and scientific committee of the faculty of medicine at Mu'tah University approved the study (approval no. 22/2/12).

2.4. Agar Well Diffusion Method

Extracts inhibition effects were assessed by agar well diffusion method according to the National Committee for Clinical laboratory Standards (NCCLS, 2000a). About 20 ml of Mueller Hinton agar (Oxoid, Hampshire, England) was poured into Petri dishes. After solidification, inoculum of 0.5 McFarland of each test strain was seeded on the media. Allowing inoculum to dry; 5 mm size wells was made with sterile borer. About 50 µl of 100 mg/ml of each extract was introduced into the well and plates were incubated at 37°C for 24 hrs. All samples were tested in duplicates. Other 2-32 mg/ml dilutions were prepared to determine the concentration effect (CE) on bacterial inhibition. Water, vancomycin (30 µg) and oxacillin (1 µg) disks were used as negative and positive controls (Abu-Shanab *et al.,* 2004). Antimicrobial effect was determined by measuring the diameter of zone of inhibition around the holes and disks.

2.5. Minimal Inhibitory Concentration (MIC) and Minimal Bactericidal Concentration (MBC) by Broth Dilution

MIC and MBC were detected by using different extract concentrations from 0.0625 mg/ml to 32 mg/ml. In a test tube equal volume (0.5 ml)of both plant extracts and nutrient broth were mixed together. Standard microbial inoculums were added to each tube (0.1 ml of $1–2 \times 10^8$ cfu/ml). Tubes were aerobically incubated at 37°C for 24 hrs according to NCCLS (2000b). Vancomycin and oxacillin dilution tubes were prepared and used as positive and negative controls in all tests.

Test tube with no visible growth (no turbidity) was considered as the lowest extract concentration that inhibits bacterial growth (MIC), whereas, tube with no visible growth that yielding no colonies when subcultured on extract or drug free nutrient agar was considered as MBC tube (Weckesser *et al.,* 2007).

3. Results

3.1. Primary Assessment of Antimicrobial Activity

Qualitatively and quantitatively antibacterial activities of *S. libanotica* and *R. officinalis* against MRSA were in vitro assessed (Table 2). Fifty micro-liters of 100 mg/ml of *R. officinalis* and *S. libanotica* showed the greater effect with MIC and MBC values range of 0.125-0.25 mg/ml and 0.25-0.5 mg/ml and 0.25-0.5 mg/ml and 0.5-1 mg/ml of each plant extract respectively (Table3).

Our results indicate that ethanol extract mixture of both *S. libanotica* and *R. officinalis* will diminish their potent antibacterial effect against MRSA with MIC and MBC increasing up to 4 to 8 mg/ml and 8 to 16 mg/ml, respectively (Table 3).16S rRNA gene was positive for all staphylococcal strains. PCR product of *MecA* gene was detected in all MRSA strains but not MSSA strains.

Table 2. Antibacterial activity of the ethanol crude plant extract on *S. aureus* strains

Plant used	MRSA[a] (n=10)	MRSA[b] (n=15)	*S. aureus and MSSA
Rosmarinus officinalis	28#	26	28
Salvia libanotica	23	22	26
Vancomycin 30µg	21	21	22
Oxacillin 1µg	NA	NA	16
Negative control (Water)	NA	NA	NA

#Inhibition zone average in mm;*S. aureus = standard strain used ATCC 29213.
a Community MRSA isolates; b Clinical MRSA isolates; n = number
Vancomycin, Oxacillin = standard antibacterial drug used as positive control of MRSA tested. NA=No activity

Table 3. MIC and MBC (mg/ml) of ethanol extracts of Rosemary and Mediterranean Sage and their combination on MRSA.

Plant used	MRSA[a] (n=10)		MRSA[b] (n=15)	
	MIC	MBC	MIC	MBC
Rosmarinus officinalis	0.125-0.25	0.25-0.5	0.25-0.5	0.5-1
Salvia libanotica	0.25-0.5	0.5-1	0.25-0.5	0.5-1
R. officinalis/ S. libanotica	4 – 8	8 – 16	4 – 8	8 – 16
Vancomycin	0.25-0.5	0.5-1	0.5-1	1-2
Oxacillin	NA	NA	NA	NA

Vancomycin, Oxacillin = standard antibacterial drug used as positive and negative controls. [a] Community MRSA isolates; [b] Clinical MRSA isolates; n = number

of MRSA tested. NA=No activity

Table 4. The concentration effect of the ethanol extracts of Rosemary and Mediterranean Sage on *S. aureus* and MRSA strains

Plant extract	Conc. (mg/ml)	Diameter (mean ± SD) of inhibition zone (mm) including well diameter of 6 mm		
		Staphylococcus aureus ATCC 29213	Community MRSA (n=10)	Clinical MRSA (n=15)
Rosemary	2	10.66 ± 1.15	8.75 ± 1.06	8.0 ± 1.04
	4	11.83 ± 0.28	10.5 ± 0.7	9.5 ± 0.1
	8	15.33 ± 1.52	14.75 ± 1.76	13.05 ± 1.5
	16	16.66 ± 0.57	17.00 ± 2.82	16.00 ± 2.5
	32	20.00 ± 0	19.00 ± 1.41	18.00 ± 1.4
Sage	2	8.16 ± 1.04	8.00 ± 0	7.8 ± 0.2
	4	10.16 ± 0.28	9.25 ± 1.06	9.1 ± 1.0
	8	16.33 ± 1.52	13.00 ± 1.41	12.00 ± 1.3
	16	17.66 ± 2.02	16.25 ± 0.35	15.5 ± 0.75
	32	19.00 ± 1.73	17.5 ± 0.7	16.2 ± 0.5

3.2. Determination of Concentration Effect (CE)

Results of CE for the bacterial samples are listed in Table 4. Ethanol extracts of both *S. libanotica* and *R. officinalis* revealed inhibitory action on *Staphylococcus aureus* ATCC 29213 and MRSA in all added doses. No significant difference in bacterial inhibition zone for both extracts was noticed for all isolates sources at any specified concentration. Nevertheless, increasing concentrations resulted in greater inhibition zone for *S. libanotica* and *R. officinalis* against all isolates.

4. Discussion

World Health Organization encouraged health systems in different countries since 1980s to interact with herbal medicine for identifying and assessing means that build up bases for new and safe herbal agents which can be used for treatment of infectious and noninfectious diseases (WHO, 1978). The development of new antibacterial drugs for the treatment of MRSA infections is of increasing interest (Schito, 2006). Herbal medicine has long been used in Jordan for the treatment of various ailments (Obeidat, 2011; Ibrahim *et al*., 2010). The incidence of MRSA infections in Jordan was obvious over the last few years (Aqel *et al*., 2012). MRSA detection rate has been increasing especially in the hospitals. The resistance has been emerging not only to methicillin but also to other many antibiotics, including vancomycin leading to further restriction on available antibiotic options (Bakri *et al*., 2007; Mohammad, 2010).

Experiments of microbial growth inhibition by agar well diffusion and broth dilution methods revealed that two plants (*S. libanotica* and *R. officinalis*) were active against MRSA strains. Similar antimicrobial results were obtained by other researchers (Abu-Shanab *et al*., 2006; Obeidat, 2011).

In this study, volatile oils and other triterpenoids, the active components determined primarily from *S. libanotica* and *R. officinalis* showed antibacterial activity against MRSA, *S. aureus* and other tested isolates, this is in agreement with previous Jordanian study by Al-Bakri *et al*. (2010). A study by Nascimento *et al*. (2000) showed that the active chemical constituents obtained from *S. libanotica* and *R. officinalis* were mainly flavonoids, phenolic acids, rosmarinic, caffeic, chlorogenic acids, carnosol, diterpenes, camphor, thuyone and cineole; all these compounds and oils have remarkable antimicrobial activity. In the current study, the MIC and MBC results revealed that low extract concentration had a bacteriostatic action, whereas bactericidal action was detected at higher concentrations, this might be due to increased intracellular uptake of the extract and more cellular damage. The antagonistic effect of the combination of ethanol extracts of *S. libanotica* and *R. officinalis* was the major finding of our study; this finding is in agreement with other previous study by Abu-Shanab *et al*. (2006). Therefore, the combination of both previous extracts which are traditionally used in Jordan against number of diseases obviously has no efficient antibacterial effect.

5. Conclusion

Our previous study by Ibrahim *et al*. (2013) showed the inhibitory action of crud extract of *S. libanotica* and *R. officinalis* against two test strains of *S. aureus;* therefore,

and depending on the previous results, we tested the antibacterial effect of *S. libanotica* and *R. officinalis* against clinical and community strains of MRSA as a second phase. The results presented here indicate that *R. officinalis* and *S. libanotica* extract preparations can be used as antistaphylococcal agent, for both MSSA and MRSA infections. *In vivo* assessment of the dose, toxicity, tolerance and clearance of the active elements of the herbal plants need more investigations.

Acknowledgement

We would like to thank Mr. Shadi Jaber and Mr. Madhar Abumurad for their technical help.

References

Abu-Shanab B, Adwan G, Abu-Safiya D, Jarrar N and Adwan K. 2004. Antibacterial Activities of some plant extracts utilized in popular medicine in Palestine. *Turkish J Biol.***28**: 99-102.

Abu-Shanab B, Adwan G, Jarrar N, Abu-Hijleh A and Adwan K. 2006. Antibacterial Activity of four plant extracts used in Palestine in folkloric medicine against methicillin-resistant *Staphylococcus aureus*. *Turkish J Biol.*,**30**: 195-198.

Ahmad I, Mehmood Z and Mohammad F.1998. Screening of some Indian medicinal plants for their antimicrobial properties. *J Ethnopharmacol.*,**62**: 183-193.

Al-Bakri AG, Othman G and Afifi FU. 2010. Determination of the antibiofilm, antiadhesive, and anti-MRSA activities of seven *Salvia* species. *Pharmacogn Mag.*, **6**:264-270.

Aqel A, Ibrahim A and Shehabi A. 2012. Rare occurrence of mupirocin resistance among clinical *Staphylococcus* isolates in Jordan. *Acta Microbiol et. Immunol Hungarica*,**59**: 239–247.

Bakri FG, Al-Hommos NA, Shehabi A, Naffa RG, Cui L and Hiramatsu K. 2007. Persistent bacteraemia due to methicillin-resistant *Staphylococcus aureus* with reduced susceptibility to vancomycin in a patient with erythrodermic psoriasis. *Scand J Infect Dis.*,**39**:457-460.

Borg MA, de Kraker M, Scicluna E, van de Sande-Bruinsma N, Tiemersma E, Monen J and Grundmann H, ARMed Project Members and Collaborators. 2007. Prevalence of methicillin-resistant *Staphylococcus aureus* (MRSA) in invasive isolates from southern and eastern Mediterranean countries. *J Antimicrobial Chemother.*,**60**: 1310-1315.

Böszörményi A, Héthelyi E, Farkas A, Horváth G, Papp N, Lemberkovics E andSzoke E.2009. Chemical and geneticrelationships among sage (*Salvia officinalis* L.) cultivars and Judean sage (*Salvia judaica* Boiss.). *J Agric Food. Chem.*,**57**:4663-4667.

Chambers HF.1997. Methicillin Resistance in *Staphylococci:* Molecular and Biochemical basis and Clinical implications. *Clin Microbiol Rev.*,**10**: 781-791.

Clinical and Laboratory Standards Institute CLSI. 2012. Performance standards for antimicrobial susceptibility testing. CLSI, Wayne, PA, twenty second informational supplement. M100-S22.

Cos P, Vlietinck AJ, Berghe DV and Maes L. 2006. Anti-infective potential of natural products: how to develop a stronger in vitro 'proof-of-concept'. *J Ethnopharmacol.*,**106**:290-302.

Emori TG and Gaynes RP. 1993. An overview of nosocomial infections, including the role of the microbiology laboratory. *Clin Microbiol Rev.*,**6**: 428-442.

Habeeb F, Shakir E, Bradbury F, Cameron P, Taravati MR, Drummond AJ, Gray AI and Ferro VA. 2007. Screening methods used to determine the anti-microbial properties of *Aloe vera* inner gel. *Methods*,**42**: 315-320.

Ibrahim A, Aqel AA and Aljamal A.2013. Effect of the methanol extracts of *Salvia libanotica, Rosmarinus officinalis, Capparis spinosa* and *Achillea fragrantissima* against two strains of *Staphylococcus aureus. Afr J Microbiol. Res.*,**7**: 3750-3753.

Ibrahim A and Aqel AA. 2010. Effect of *Salvia triloba* L. f. extracts on neoplastic cell lines. *Jordan J Biol Sci.*,**3**: 1-7.

Mohammad A. 2010. Bacteremia among Jordanian children at Princess Rahmah Hospital: Pathogens and antimicrobial susceptibility patterns. *Iranian J Microbiol.*,**2**: 22-26.

Naimi TS, LeDell KH, Como-Sabetti K, Borchardt SM, Boxrud DJ, Etienne J, Johnson SK, Vandenesch F, Fridkin S, O'Boyle C, Danila RN and Lynfield R.. 2003. Comparison of community and health care-associated methicillin-resistant *Staphylococcus aureus* infection. *JAMA*, **290**:2976–2984.

Nascimento GF, Locatelli J, Freitas PC and Silva GL. 2000. Antibacterial activity of plant extracts and phytochemicals on antibiotic-resistant bacteria. *Braz J Microbiol.*,**31**: 247-256.

National Committee for Clinical laboratory Standards. 2000a. Performance standards for antimicrobial disk susceptibility tests. 7th ed. Villanova, PA: NCCLS, Approved Standard: M2-A7.

National Committee for Clinical laboratory Standards. 2000b. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically. 5th ed. Villanova, PA: NCCLS, Approved Standard: M7-A5.

Obeidat M. 2011. Antimicrobial activity of some medicinal plants against multidrug resistant skin pathogens. *J Medicinal Plants Res.*,**5**: 3856-3860.

Petinaki E, Miriagou V, Tzouvelekis LS, Pournaras S, Hatzi F and Kontos F., Bacterial Resistance Study Group of Thessaly.2001. Methicillin-resistant *Staphylococcus aureus* in the hospitals of central Greece. *Inter J Antimicrob Agents*,**18**: 61–65.

Schito GC. 2006. The importance of the development of antibiotic resistance in *Staphylococcus aureus*. *Clin Microbiol Infect.*,**1**: 3-8.

Weckesser S, Engel K, Simon-Haarhaus B, Wittmer A, Pelz K and Schempp CM. 2007. Screening of plant extracts for antimicrobial activity against bacteria and yeasts with dermatological relevance. *Phytomedicine*, **14**: 508-516.

World Health Organization (WHO), author. 1978. The promotion and development of traditional medicine. Geneva: WHO; Technical Report Series. P: 622.

JJBS

Volume 7, Number 3, September .2014
ISSN 1995-6673
Pages 165 - 169

Jordan Journal of Biological Sciences

Molecular Identification of *Trametes* Species Collected from Ondo and Oyo States, Nigeria

Oyetayo V. Olusegun*

Department of Microbiology, Federal University of Technology, Akure, Nigeria

Received: March 14, 2014 *Revised: May 3, 2014* *Accepted: May10, 2014*

Abstract

Internal Transcribed Spacer (ITS) region of the rDNA was used in the identification of *Trametes* species collected from Nigeria. Ribosomal DNA of *Trametes* species were extracted using CTAB lysis method. The extracted DNAs were amplified using ITS region and the amplicons sequenced. BLAST search on NCBI GenBank revealed that *Trametes* species from Nigeria are more related to *T. lactinea, T. elegans, T. polyzona, T. cingulata* and *T. ijubarskii* with percentage relationship of 96 to 99%. Phylogenetic tree generated from *Trametes* sequences from Nigeria and sequences obtained from NCBI GenBank revealed 7 clades, out of which *Trametes* from Nigeria were placed in 3 separate clades. This study showed that most of the gene sequences of *Trametes* species indigenous to Nigeria are not 100% homologous with existing gene sequence found in NCBI GenBank. The closest *Trametes* species to the *Trametes* species indigenous to Nigeria are *T. lactinea* and *T. polyzona* with 99% level of similarity.

Keywords: *Trametes* species, Nigeria, rDNA, ITS.

1. Introduction

For centuries, mushrooms have been appreciated as sources of food nutrients and pharmacologically important compounds useful in medicine. Mushrooms are known to be medically active in several therapies, such as antioxidant, antitumor, antibacterial, antiviral, hematological and immunomodulation (Wasser and Weis, 1999; Lindequist *et al*., 2005). The above health promoting properties of mushrooms have been attributed to the presence of some bioactive compounds such as glycolipids, compounds derived from shikimic acid, aromatic phenols, fatty acid derivatives, polyacetylamine, polyketides, nucleosides, sesterterpenes, polysaccharides and many other substances of different origins (Lorenzen and Anke, 1998; Wasser and Weis, 1999; Mizuno, 1999; Liu, 2007).

Mushroom belonging to the following genera; *Ganoderma, Tremella, Fuciformis, Lentinus, Grifola, Schizophyllum, Trametes, Cordyceps,* and some others had been used in treating various ailments (Oyetayo, 2011). Both cellular components and secondary metabolites of a large number of mushrooms have been shown to boost the immune system of the host and, therefore, could be used to treat a variety of diseases (Wasser and Weis, 1999).

In the last three decades, attention had been paid to the myconutraceutical potentials of macrofungi. It has been estimated that about 140,000 mushrooms are on earth, but only 14,000 (10%) are known. A large number of the unknown species exists in major parts of Africa because there are no records available on mushrooms that are indigenous to this part of the globe. Identification of mushrooms is mainly done by morphological description of the fruiting bodies, host specificity, and geographical distribution (Seo and Kirk, 2000). In most cases, morphological characteristics have their limitations in allowing a reliable distinction of intraspecific characteristics.

Trametes Fr. consist of polyporoid white rot fungi (Tomsosky *et al*., 2006). This genus is distinguished by a pileate basidiocarp, di- to trimitic hypha systems and a smooth non-dextroid spores (Ryvarden, 1991). It is widespread in distribution and consists of about fifty species (Kirk *et al*., 2008). Some species of *Trametes* has been used in medicine in China (Cui *et al*., 2011). A *β*-glucans, krestin from cultured mycelia biomass of *T. versicolor* (Turkey Tail) had been reported to possess antitumor activity (Ikekawa 2001; Wasser, 2002).Antioxidant property of extracts from *T. versicolor* collected from Nigeria had also been reported (Oyetayo*et al*., 2013). In the preparation of medicinal mushrooms as functional health-aid and a nutritional supplement as well, correct identification and quality control is essential (Lee

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

et al., 2006).Species constituting the genus *Trametes* are similar in morphology, hence it is difficult to identify and separate these species based on traditional taxonomy (Zhang *et al*., 2006).

Molecular techniques could be used to adequately characterize and identify intra and inter specific characteristics (Zakaria*et al*., 2009).The present study is, therefore, on the molecular characterization of wild *Trametes* species collected from Ibadan and Akure, Nigeria using ITS region of rDNA. Moreover, the phylogenetic relationship of the wild *Trametes* species was compared with existing *Trametes* sequences obtained from NCBI GenBank was also ascertained.

2. Materials and Methods

2.1. Fungal Material

Fruit bodies of Trametes species were collected from Oyo and Ondo States, Nigeria, between September 2012 and July 2013. The fruit bodies were kept dry in tissue papers that were placed in a polythene paper containing silica gel. The polythene bags containing the samples were well labeled for easy identification and taken to the laboratory for further examination. Herbarium samples of Trametes species fruit bodies were kept at the herbarium of Institute of Microbiology, Chinese Academy of Sciences, Beijing.

2.2. Extraction of DNA

Standard DNA isolation methods employing Cetyltrimethyl ammonium(CTAB) lysis buffer (Zolan and Pukkila 1986) were used. Briefly, dried portions of *Trametes* fruit bodies (2g) were ground with a mortar and pestle. The grounded materials were transferred into well labeled microtubes. Pre-warmed (600C) extraction buffer (CTAB) was added and the tubes were incubated at 650 C for 30 to 60 min. Equal volume of chloroform and alcohol (24:1) was added and mixed by inverting tubes for 15 min. The tubes were centrifuged for 10 min at 10,000 g (13000 rpm). The process was repeated, but the time of mixing was 3 min and the time of centrifugation was 5 min at the same speed referred to above. Upper aqueous layers were removed into clean tubes and 40µl Sodium acetate (NaAc) was added followed by 260µl of cold isopropanol. This was gently mixed by inverting tubes. The tubes were incubated at -200 C overnight. On the second day, the mixture was centrifuged at 10,000 g (13000 rpm) for 10 min. The supernatant was discarded and pellets rinsed with 70% alcohol and mixed for sometimes. This procedure was repeated three times. After discarding the supernatant, the sample was dried in a dryer for 20 min at room temperature. Pellets were resuspended in 30µl of Tris EDTA (TE) buffer. DNA concentration and quality were checked by observing the band on an ethidium-stained agarose gel (0.7%) using 0.2µl of each sample.

2.3. PCR Amplification of the ITS Region

The entire region of rDNA of *Trametes* species was amplified by PCR using the primers, ITS4 and ITS5. The reaction mix was made up to a total volume of 25 µl, composed of 23 µl of Taq polymerase "Ready to Go" mixture (Pharmacia, Sweden) with 0.2 µl of each primer (100 pM) and 2 µl of DNA solution. The tubes were placed in a thermal cycler (GenAmp PCR System 2400, Perkin–Elmer, USA) for amplification under the following conditions: 30 cycles of (1) denaturation at 95°C for 30 s, (2) annealing at 50°C for 1 min, (3) extension at 72°C for 1 min. The amplification products were purified using a PCR Purification Kit (USA) and electrophoresed on ethidium-stained agarose gel (0.7%) to check the purity. DNA sequencing was performed using the same primer pair used in the PCR reactions (ITS 4 and ITS 5) in an Applied Biosystem DNA Analyser (USA).

2.4. Alignment of Sequence

Alignments were performed with the Clustal W package (Thompson et al., 1997). The aligned sequences were corrected manually and through focusing on gap positions. DNA sequence data were analyzed to provide pairwise percentage sequence divergence. The data obtained from the sequence alignment were used to plot a tree diagram (MEGA 4 Software).

3. Results

Internal transcribed spacer (ITS) region of the nuclear rDNA sequences of the 19 *Trametes* specimens was used for molecular identification. The ITS discriminated between all the *Trametes* species collected from Ondo and Oyo States, Nigeria and *Trametes* species sequences obtained from NCBI GenBank (Table 1). The level of relatedness of *Trametes* species collected in Nigeria and the already existing gene sequences of *Trametes* species in NCBI GenBank ranges between 96 to 99% (Table 1).

The closest relatives of *Trametes* species designated specimens 1 to 19 collected from Nigeria were *Tramete slactinea,T. elegans*, *T. polyzona*, *T. cingulata* and *T. ijubarskii* (Table 1).

Phylogenetic tree generated from the gene sequence of *Trametes* species collected from Nigeria and the sequences from NCBI GenBank showed a marked difference. A total of 8 clades were generated in the final phylogenetic tree (Figure 1). A clade is made up of organisms from the same ancestral stock (Dupuis, 1984). Clade 1 has *Trametes* species collected from Nigeria and designated specimens 5, 12, 18, 24, 25 and 26 placed alongside macrofungi such as *Trametes lactinea, Trametes elegans, Trametes cubensis, Lenzites elegans* and *Pycnoporus sanguineus.* Clade 2 is made up of made up of *Trametes cubensis* (JN164923.1); *Trametes* sp. (HQ916734.1); *Lenzitese legans* (HQ248217.1) is placed in clade 3 while clades 4 and 5 are made up of *Trametes menziesii* (JN645071.1) and four *Trametes sanguine*(with ascension numbers JN164981.1, JN164982.1, KC243780.1, JX082366.1) respectively. Three separate clades (6, 7 and 8) were observed for *Trametes* species collected in Nigeria.

Table 1. Genomic identification of *Trametes* species collected from Nigeria based on ITS Region of rDNA

Specimen	Tentative Identity	Closest Relative	Accession Number	% Level of Closeness
1	*Trametes* sp	*Trametes polyzona*	JN164978.1	99
2	*Trametes* sp	*Trametes ijubarskii*	AY684174.2	96
3	*Trametes* sp	*Trametes lactinea*	JN048769.1	99
4	*Trametes* sp	*Trametes polyzona*	JN164980.1	99
5	*Trametes* sp	*Trametes* l*actinea*	JN048769.1	99
6	*Trametes* sp	*Trametes polyzona*	JN164980.1	99
7	*Trametes* sp	*Trametes lactinea*	JN048769.1	99
8	*Trametes* sp	*Trametes elegans*	JN164921.1	98
9	*Trametes* sp	*Trametes elegans*	JN164921.1	98
10	*Trametes* sp	*Trametes elegans*	JN048766.1	98
11	*Trametes* sp	*Trametes lactinea*	JN048769.1	99
12	*Trametes* sp	*Trametes lactinea*	JN048769.1	99
13	*Trametes* sp	*Trametes lactinea*	JN048769.1	99
14	*Trametes* sp	*Trametes polyzona*	JN164978.1	99
15	*Trametes* sp	*Trametes elegans*	JN048766.1	98
16	*Trametes* sp	*Trametes elegans*	JN164978.1	99
17	*Trametes* sp	*Trametes polyzona*	JN164978.1	99
18	*Trametes* sp	*Trametes elegans*	JN048766.1	98
19	*Trametes* sp	*Trametes cingulata*	JN645075.1	98

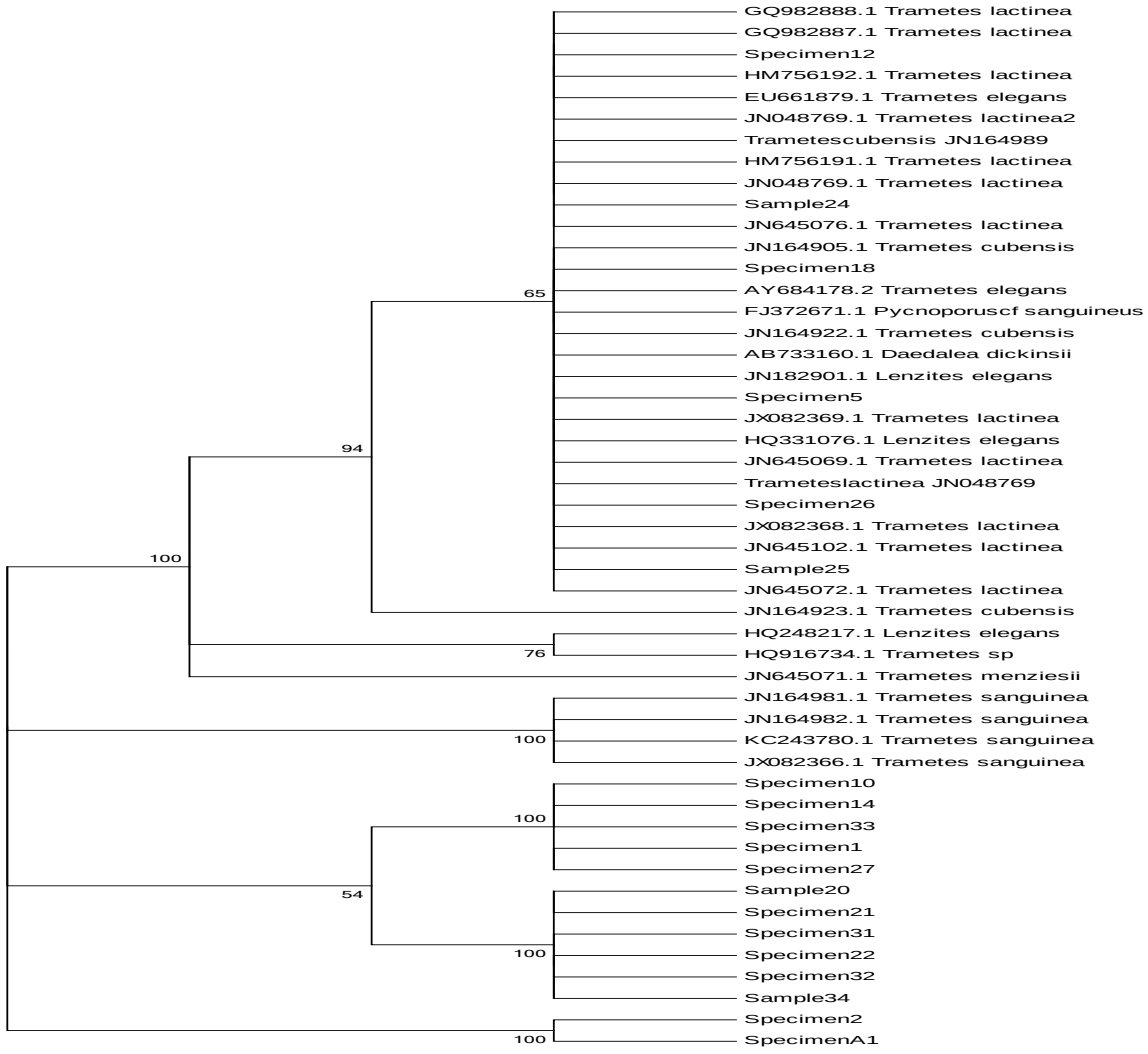

Figure 1. Phylogenetic tree of the genus *Trametes* species collected from Nigeria based on sequences of the ITS Region

4. Discussion

Trametes is regarded as one of the most confused group of genera in Polyporaceae (Cui *et al*., 2011). Zhang *et al*. (2006) had earlier questioned the current taxonomy of *Trametes*. Hence, mycologists have attempted to use sequence data to resolve the taxonomic problems in *Trametes* and in the related genera (Ko and Jung, 1999; Tomšovský *et al*., 2006; Zhang *et al*., 2006; Miettinen and Larsson, 2010). Analysis of the ITS region of the nuclear rDNA of *Trametes* species collected from Nigeria revealed the genetic difference in the 19 *Trametes* species and the *Trametes* species sequences obtained from NCBI GenBank (Table 1). The ITS region of rDNA has been reported to be the most used genomic region for molecular characterization of fungi (Gardes and Bruns, 1993). The ITS region of Nuclear rDNA has several characteristics making it a pertinent tool to identify and analyse phylogenetic molecules of fungi at species level (Anderson and Stasovski, 1992; Gardes and Bruns, 1993).ITS sequences are useful in distinguishing the genera with similar morphological characteristics (Cui *et al*., 2011). Moreover, the core structure of ITS is conserved in the mature rRNA molecules, and it is much diverse in both sequence and size (Lalev and Nazar, 1998).

Trametes species designated specimens 1, 2, 4, 6, 8, 9, 10, 14, 15, 16, 17, 18 and 19 were placed in three different clades. This shows that the macrofungi are not from the same ancestral stock with *Trametes* species whose sequences are already in NCBI GenBank. In an earlier study on the phylogeny of European and one American species of the genus *Trametes*, all *Trametes* species except *T. cervina* were reported to form a clade (Tomsovsky *et al*., 2006). Similarly, in a phylogenetic tree generated by Zhang *et al*. (2006) *Trametes* species was separated from other groups and most strains of *T. versicolor* formed a single clade with a high percentage support. However, *Trametes* species designated specimens 3, 5, 7, 11, 12 and 13 were placed in the same clade with *T. lactinea* and *T. cubensis*. This shows that they are more related to these two species.

This study revealed that most of the gene sequences of *Trametes* species indigenous to Nigeria are not 100% homologous with existing gene sequence found in NCBI GenBank. The difference in the gene sequences of *Trametes* species from Nigeria and its counterpart from other parts of the world maybe due to the different ecological zones where they exist. In a recent report, Wu *et al*. (2013) stated that geographic distance is the dominant factor driving variation in fungal diversity at a regional scale (1000–4000 km), where as environmental factors (total potassium and total nitrogen) explain variation in fungal diversity at a local scale (<1000 km). The closest *Trametes* species to the *Trametes* species indigenous to Nigeria are *T. lactinea* and *T. polyzona* with 99% level of similarity.

Acknowledgement

The author gratefully acknowledges CAS-TWAS for award of visiting Scholar Fellowship to China. Prof. Y.-J, Yao isalso acknowledged for hosting Oyetayo, V.O in his laboratory (Key Laboratory of Systematic Mycology and Lichenology, Institute of Microbiology, Chinese Academy of Sciences, Beijing 100101, Peoples' Replublic of China).

References

Anderson JB and Stasovski E. 1992. Molecular phylogeny of Northern Hemisphere species of *Armillaria*. *Mycol*.,**84:** 505–516.

Cui DZ, Zhao M, Yang HY, Wang Cl and Dai HB. 2011. Molecular phylogenyof *Trametes* and related genera based on internal transcribed spacer (ITS) and nearly complete mitochondrial small subunit ribosomal DNA (mt SSU rDNA) sequences.*Afri J Biotech*.,**10**(79): 18111-18121.

Dupuis C. 1984. "Willi Hennig'simpact on taxonomic thought". *Annual Rev Ecol Systematics,***15**: 1–24.

Gardes M and Bruns TD.1991. Rapid characterization of ectomycorrhizae using RFLP pattern of their PCR amplified-ITS. *Mycol Soc News Let.*,**41**: 44–45.

Ikekawa T. 2001. Beneficial effects of edible and medicinal mushrooms in health care. *Int. J Med Mushrooms*,**3**:291–298.

Kirk PM, Cannon PF, Minter DW and Stalpers JA. 2008. **Dictionary of the Fungi** (10th ed.). Wallingford, UK: CAB International. p. 695.

Ko KS and Jung HS. 1999. Molecular phylogeny of *Trametes* and related genera. Anton Leeuw Int J G, **75**: 191-199.

Lalev AI and Nazar RN. 1998. Conserved core structure in the internal transcribed spacer 1 of the *Schizosaccharomyces pombe* precursor ribosomal RNA. *J Mol Biol*.,**284**:1341-1351.

Lee JS, Linz MO, Cho KY, Cho JH, Chang SY and Nam DH. 2006. Identification of medicinal mushroom species based on nuclear large subunit rDNA sequences. *J Microbiol.*, **44** (1):.29-34.

Lindequist U, Niedermeyer THJ and Julich WD. 2005. The pharmacological potential of mushrooms-Review. *E CAM*, **2** (3): 285 – 299.

Lorenzen K and Anke T. 1998. Basidiomycetes as a source for new bioactive natural products. *Current OrganicChem.*, **2**:329-64.

Liu J-K. 2007. Secondary metabolites from higher fungi in China and their biological activity. *Drug Discov Ther*., **1(2):** 94 – 103.

Miettinen O and Larsson KH.2010. *Sidera*, a new genus in Hymenochaetales with poroid and hydnoid species. *Mycol Progress*, **10**:131-141.

Mizuno T. 1999.The extraction and development of antitumor active polysaccharides from medicinal mushrooms in Japan—review. *Int J Med Mushr*., **1**: 9–30.

Oyetayo VO. 2011. Medicinal uses of mushrooms in Nigeria: Towards full and sustainable exploitation. *Afr J Tradit Complement Altern Med*., **8 (3):** 267-274.

Oyetayo VO, Nieto- Camacho A, Ramırez-Apana TM, Baldomero RE and JimenezM. 2013. Total phenol, antioxidant and cytotoxic properties of wild macrofungi collected from Akure Southwest Nigeria. *Jordan J Biol Sci.*,**6**(2): 105 – 110.

Ryvarden L. 1991. **Genera of Polypores. Nomenclature and Taxonomy**. Synopsis fungorum 5. Fungiflora, Oslo, Norway, 363 pp.

Seo GS and Kirk PM. 2000.Ganodermataceae: Nomenclature and classification, In: Flood, J., P.D. Bridge and P. Holderness

(Eds.),**Ganoderma Disease of Perennial Crops**. CABI Publishing, Walling Ford, UK., pp. 3 – 22.

Thomson JD, Gibson TJ, Plewniak F, Jeanmougin F and Higgins DG, 1997. The Clustal_X windows interface: Flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res.*,**25**: 4876–4882.

Tomšovský M, Kolaĺík M, Sylvie Pañoutová S and Homolka L. 2006.Molecular phylogeny of European *Trametes* (Basidiomycetes, Polyporales) species based on LSU and ITS (nrDNA) sequences. Verlagsbuchhandlung, D-14129 Berlin · D-

70176 Stuttgart .

Wasser SP and Weis AL. 1999. Therapeutic effects of substances occurring in higher basidiomycetes mushrooms a modern perspective. *Crit Rev Immunol.,* **19** (1)*:* 65-96.

Wasser SP. 2002. Medicinal mushrooms as a source of antitumour and immunostimulating polysaccharides. *Appl Microbiol Biotechnol.,* **60**: 258-274.

Wu B, Tian J,Bai C, Xiang M, Sun J and Liu X. 2013.The biogeography of Fungal communities in wetland sediments along the Changjiang River and other sites in China. *The ISME J.*,**7:** 1299–1309.

Zakaria L, Ali NS, Salleh B and Zakaria M. 2009. Molecular analysis of *Ganoderma* species from different host in Peninsula Malaysia. *J Biol Sci.,* **9**(1)*:* 12 – 20.

Zhang X, Yuan J, Xiao Y, Hong Y and Tang C. 2006.A primary studies on molecular taxonomy of Trametes species based on the ITS sequences of rDNA. *Mycosystema*, **25**(1):23-30.

ZolanME and Pukkila PJ. 1986.Inheritence of DNA methylation in *Coprinus cinereus*. *Mol Cell Biol.,* **6**: 195-200.

JJBS

Volume 7, Number 3, September .2014
ISSN 1995-6673
Pages 171- 177

Jordan Journal of Biological Sciences

Coronary Atherosclerosis: Adiponectin and Leptin as Predictors of Disease Severity

Samir S. Mahgoub[1,*], Ayman J. Hammoudeh[2], Hani M. Al-shagahin[3], Manal N. Al S,oub[4], Odai F. Masarweh[5], Moustafa A. Abdo[5], Shereen N. AlAtoom[5] and Areej W. Alzaraq[5]

[1]Biochemistry and Molecular Biology Department, Faculty of Medicine, [2] Interventional Cardiology Department, Istishari Hospital, Amman, [3] Otolaryngology Department,[4] Management Department, Faculty of Pharmacy,

[5] Sixth year students, Faculty of Medicine, Mu'tah University, Al Karak, Jordan

Received: April 10, 2014 Revised: May 11, 2014 Accepted: May 15, 2014

Abstract

Adipose tissue is known to produce and release numerous bioactive substances, known as adipokines (such as leptin and adiponectin), which have been found to be involved in various physiological processes, including the regulation of arterial tone and they are related to cardiovascular risk factors. The objective of the present study was to determine the relationship between the levels of serum leptin and adiponectin and the degree of coronary heart disease, also, to compare the sensitivity and specificity of serum circulating levels of the these two biomarkers in CAD diagnosis. Forty nine patients with established coronary artery disease (CAD) defined as old myocardial infarction and angina pectoris classified as CAD group. The control group included twenty normal healthy subjects. All patients and controls were subjected to complete clinical history taking, clinical examination including 12 lead electrocardiograms (ECG), diagnostic coronary angiography (CA) and the colorimetric measurement of serum levels of triacylglycerols (TGs), total cholesterol (total-C), low density lipoprotein-cholesterol (LDL-C), high density lipoprotein-cholesterol (HDL-C), also, ELISA for measurement of leptin and adiponectin. The predictors of coronary atherosclerosis severity include higher LDL-C, low serum adiponectin level, higher leptin level and previous myocardial infarction. Serum levels of leptin, LDL-C and total-C showed highly significant ($p< 0.0001$) increase, while, adiponectin levels showed highly significant ($p< 0.0001$) decrease in the group of patients when compared to the levels of the control group. The levels of HDL-C in the group of patients were significantly ($p< 0.05$) lower than in the control group. There was no significant difference between the levels of TGs in the patients versus the controls. The levels of leptin showed negatively significant correlation with the levels of adiponectin ($r=0.76$, $p<0.001$), it was positively significant with the levels of LDL-C ($r=0.302$, $p=0.035$), while, there was no significant correlation between the levels of leptin and HDL-C and the levels of adiponectin and HDL-C, there was a weak but significant correlation between the levels of serum adiponectin and LDL-C ($r=0.2$, $p=0.001$). The overall positive rates obtained from Receiver Operating Characteristic (ROC) curve for evolution of sensitivity and specificity of the different biomarkers is obtained. The sensitivity was 100% for both leptin and adiponectin. ROC curve results revealed that the specificity for leptin and adiponectin were 100% and 90%, respectively. The results obtained in the present study indicate that serum leptin and adiponectin might play an important pathogenic role not only in the occurrence but also in the severity of CAD. The circulating level of leptin provides highly specific biomarker for CAD more than adiponectin.

Keywords: Adiponectin, Leptin, Coronary Artery Disease

1. Introduction

White adipose tissue stores excess energy in the form of triglycerides, while brown adipose tissue is actively involved in the regulation of body temperature (Mariman and Wang, 2010). Recent studies have shown that adipose tissue is an active endocrine and paracrine organ secreting several mediators called adipokines.

Adipokines include hormones, inflammatory cytokines and other proteins (Nele and Johan, 2011). These adipokines include hormones as leptin and adiponectin, inflammatory cytokines as tumor necrosis factor α, interleukin-6 and other proteins as plasminogen activator inhibitor-1, angiotensinogen and resistin (Wozniak *et al*., 2009).

Furthermore, adipose tissue is known to release an unidentified adipocyte-derived relaxing factor (Löhn *et al*., 2002), which relaxes several arteries. Leptin is an ob

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

gene-expressed protein mainly secreted by adipose tissues, with a primary role of inhibiting food intake, modulating weight balance and promoting energy metabolism (Brubeck, 2006).

Previous research has revealed that leptin is a stress mediator after injuries, and it proceeds to maintain homeostasis by accelerating oxidation of glucose and fatty acids, alleviating reactive oxygen species-induced apoptosis, and ameliorating post-septic multiple organ dysfunction (Eguchi *et al*., 2008, Lin *et al*., 2007).

Several experimental studies have shown that increased leptin level may directly or indirectly exert multiple actions at the cardiovascular level (Beltowski, 2006), where leptin receptors have been identified in various peripheral tissues, including the cardiovascular system and in human coronaries; it seems to have both vasodilatory and vasoconstrictory actions on vascular smooth muscle (Quehenberger *et al*., 2002).

Furthermore, leptin is involved in a number of diverse physiological processes, such as regulation of endocrine functions, inflammation, immune response, reproduction and angiogenesis (Otero *et al*., 2005). Several studies have found a significant association between circulating plasma leptin with insulin resistance and inflammatory markers, suggesting leptin as a risk factor for cardiovascular disease (Van Dielen *et al*., 2001).

Adiponectin is a protein hormone secreted by adipocytes; it binds to two different seven transmembrane domain receptors called AdipoR1 and AdipoR2. AdipoR1 is predominantly expressed in skeletal muscles, whereas AdipoR2 is predominantly expressed in liver and throughout the brain (Bjursell *et al*., 2007). Many other cells have adiponectin receptors as macrophages, osteoblasts, adipocytes, endothelial and muscular cells of the vascular wall, pancreatic cells and central nervous system (Zhou *et al*., 2005).

Adiponectin has been considered an anti-inflammatory and antioxidative adipokine that protects against cardiovascular disease (Antoniades *et al*., 2009). Plasma adiponectin has been correlated with endothelium-dependent vasorelaxation in humans (Tan *et al*., 2004). These results were confirmed by other studies that have shown an increase in NO production as well as NO-mediated and potassium channel-mediated (voltage-dependent) vasorelaxation in rats by adiponectin (Greenstein *et al*., 2009, Xi *et al*., 2005, Fésüs *et al*., 2007). Increased NO production inhibits platelet aggregation, leucocyte adhesion to endothelial cells and vascular smooth muscle cell proliferation. Furthermore, it reduces oxidative stress by decreasing ROS production in endothelial cells. All of these effects protect the vascular system against endothelial dysfunction (Antoniades *et al*., 2009).

The aim of the present study is to determine the relationship between the levels of serum leptin & adiponectin and the degree of coronary heart disease; also, to compare the sensitivity and specificity of serum circulating levels of these two biomarkers in CAD diagnosis.

2. Materials and Methods

2.1. Patients And Study Protocol

The criteria for the diagnosis of CAD include myocardial infarction and angina pectoris based on the clinical history, ECG and diagnostic coronary angiography (CA) was carried out on forty nine consecutive patients with age ranging between 50-65 years with mean ±SD of 59.175±3.112 years (31 males and 18 females) who were selected from the Interventional Cardiology Department, Istishari Hospital to participate in the current study, the duration between the onset of disease and the time of performing the assay of the biomarkers was ranging between 90-270 days with mean ±SD of 136.48±4.96 day. The control group included 10 normal healthy subjects with age ranging between 54-61 years with mean ±SD of 57.200±2.573 years (17 males and 3 females) who were non-diabetic, non-hypertensive, with no history of previous CAD; having normal ECG and normal (CA). A written informed consent was obtained from each participant. All patients and the control groups were subjected to diagnostic coronary angiography (CA) in Cath-Lab of Interventional Cardiology Department, Istishari Hospital and the biochemical analyses were carried out in the Biochemistry and Molecular Biology Department, Faculty of Medicine, Mu'tah University.

2.2. Diagnostic Coronary Angiography (CA)

It was done for all participants using a flat-panel imaging system. All subjects were fasting and sedated. It was performed from the femoral artery approach. After local groin infiltration of 10-20 ml xylocaine 2% using modified seldinger's technique and injection of 5000 IU of Heparin, 6F JL then JR coronary catheters were used to engage the corresponding arteries. The study was conducted with a General Electric Innova 2000 angiographic unit (GE medical system Milwaukee, WI, USA). The selection criteria of the patients were presence of more than 50% of coronary lesions in their angiographic projections and normal (CA) to be used as a control group.

2.3. Laboratory Measurements

Blood samples were drawn after an overnight fast from each patient of the test group and each healthy subject of the control group. Each blood sample was centrifuged to collect serum, which was stored at -20oC till the time of analysis. Total-C, HDL-C and TGs were measured by enzymatic colorimetric methods as described by Richmond (1973), Gordon et al. (1977) and Jacobs and Vandemark (1960), respectively, using reagents from (Human Gesellschaft fur Biochemica Diagnostica mbH, Germany).

LDL-C was calculated by Friedewald's formula (Friedewald *et al.*, 1972). Leptin was measured using Human Leptin ELISA kit (SRL, Tokyo) and adiponectin was estimated using Human Adiponectin ELISA kit (Otsuka Pharmaceutical Inc., Tokyo), as described by Engvall *et al.* (1971).

2.4. Statistical Analysis

All data were analyzed using analysis of variance (ANOVA) test for the comparison between the different means of variables and the data summarized as mean and standard deviation (mean± SD). Correlation between different numerical variables was done using Spearman correlation test (r). Differences were accepted as significant at $p<0.05$. ROC curve analysis was done using MedCalc software for evolution of sensitivity and specificity of the different biomarkers.

3. Results

The biochemical parameters of the patients' group versus the control group are presented in Table 1, in the form of mean ±SD. The results showed highly significant ($p< 0.0001$) increase in the levels of leptin, LDL-C and total-C of the CAD group versus the control group, also, there was highly significant ($p< 0.0001$) decrease in the levels of adiponectin of patients when compared to the controls. HDL-C values revealed a significant ($p< 0.05$) decrease for CAD group in respect to the control group, while, the values of TGs showed insignificant difference ($p=0.0871$).

Table 1. Baseline biochemical parameters of CHD and control groups

Parameter	CAD group (n=49)	Control group (n=20)
Leptin (ng/mL)[a]	27.72±4.28*	12.75±1.72
Adiponectin (µg/dL)[a]	7.23±1.01*	12.12±1.14
TGs (mg/dL)[a]	298.562±30.34**	237.95±8.73
LDL-C (mg/dL)[a]	148.24±6.16*	105.09±5.32
HDL-C (mg/dL)[a]	32.10±2.07***	37.18±3.24
Total-C (mg/dL)[a]	289.37±23.68*	187.31±2.38

[a] Values were expressed as mean ± standard deviation (SD), *=*P*<0.0001is highly significant, **=*P*>0.05is insignificant and ***=*P*<0.05is significant when compared with the values of the control group.

In CAD group, the obtained results revealed a negatively significant correlation between the levels of serum leptin and adiponectin (r=0.76, $p<0.001$), positively significant correlation between the levels of serum leptin and LDL-C (r=0.302, $p=0.035$), while, there was no significant correlation between levels of leptin and HDL-C (r=0.011, $p=0.94$) & the levels of adiponectin and HDL-C (r=0.007, $p=0.96$), there was a weak significant correlation between the levels of serum adiponectin and LDL-C (r=0.2, $p=0.001$) (Figure 1).

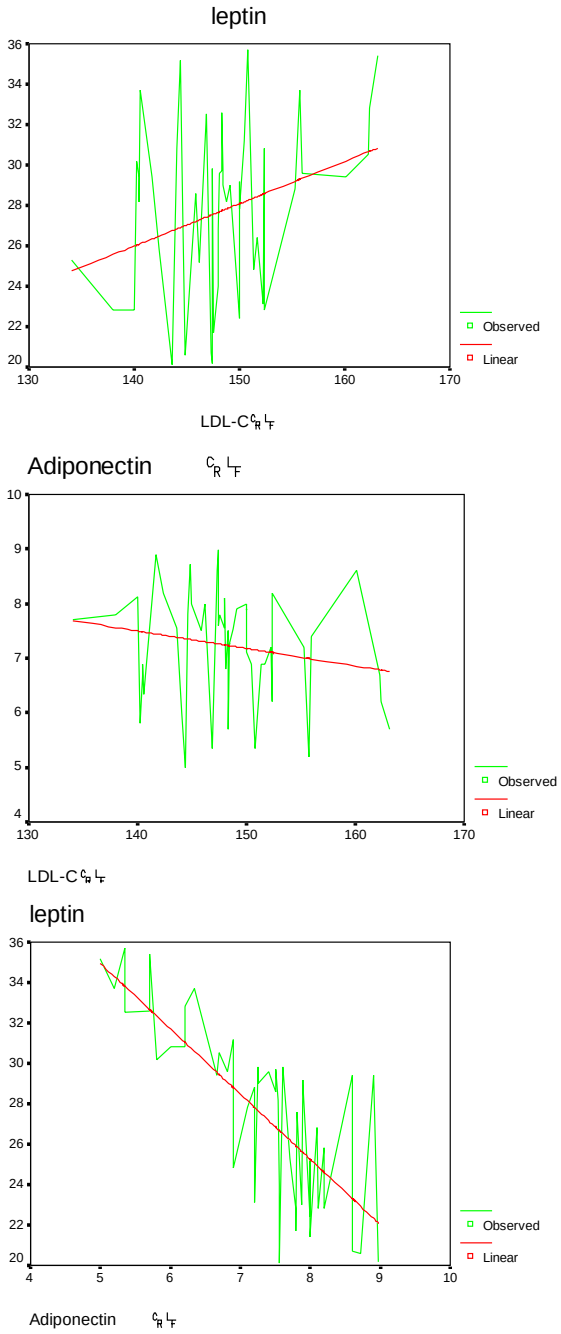

Figure 1. Correlations between some of the biochemical parameters of the study (CAD) group

Table 2 shows the area under the ROC curves for leptin and adiponectin in (1.00 and 0.00 for the two parameters, respectively). Also, the optimal cutoff value of leptin (27.7 ng/mL) (sensitivity 100% and specificity 100%) (Figure 2), of adiponectin (7.6µg/dL) (sensitivity 100% and specificity 90%) (Figure 3)

Table 2. Area under the (ROC) curves for the two parameters

Test Result Variable(s)	Area	Asymptotic 95% Confidence Interval	
		Lower Bound	Upper Bound
Leptin	1.000	1.000	1.000
Adiponectin	0.000	0.000	0.000

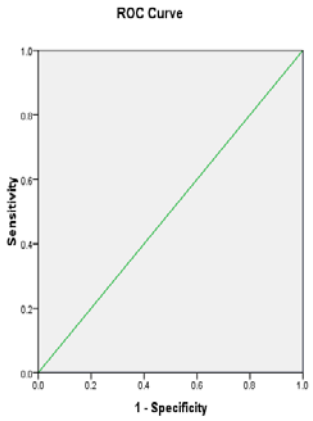

Figure 2.ROC curve for leptin.

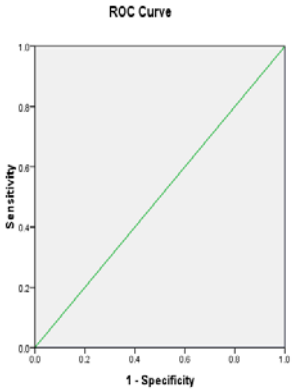

Figure 3. ROC curve for adiponectin

4. Discussion

Leptin and adiponectin differ from almost all other adipocytokines in being secreted exclusively by adipocytes, the details of all the factors regulating their synthesis, secretion and clearance remain incomplete (Finucane *et al*., 2009).

Adiponectin is a 244 amino acid protein (Sattar *et al*., 2009). It has been shown to have several beneficial effects in the cardiovascular system including an essential role in the maintenance of heart architecture, as the cytokine may attenuate angiotensin II-induced cardiac hypertrophy (Shibata *et al*., 2004**)**. Also, it represses atherosclerotic lesions in a mouse model of atherosclerosis and adiponectin-deficient mice exhibit an accelerated vascular remodeling response to injury (Ouchi *et al*., 2003).

In addition, adiponectin stimulates nitric oxide production in endothelial cells through AMPK-dependent and AMPK-independent phosphorylation of endothelial nitric oxide synthase (eNOS) (Cheng *et al*., 2007) and hypoadiponectinemia is associated with the progression of left ventricular hypertrophy (LVH), which is accompanied by diastolic dysfunction (Hong *et al*., 2004).

Through the induction of cAMP-activated protein kinase, adiponectin can stimulate glucose uptake by muscles, fatty acids oxidation in muscles and liver, also, decrease hepatic glucose production, cholesterol and triacylglycerols synthesis and lipogenesis (Ouchi *et al*., 2000). Therefore, increased blood lipid concentrations in this study may be explained by our results which showed decreased adiponectin concentrations in the CAD group of patients.

Although whether low levels of adiponectin predict hypertension remains controversial (Asferg *et al*., 2010) and whether adiponectin levels in hypertension are decreased (Adamczak *et al*., 2003), low adiponectin levels might contribute to the pathogenesis of obesity-related hypertension.

This study confirms the previous reports (Hara *et al*., 2007, Selcuk *et al*., 2008) that plasma adiponectin levels are lower in patients with CAD and correlated significantly to the severity of disease. However, Lim *et al*. (2005) found no significant relation between serum adiponectin and the severity of coronary atherosclerosis. Studies in experimental animals have shown that adiponectin has the potential to inhibit neointimal formation (Jaleel *et al*., 2006), which is supported by the report of (Kubota *et al*., 2002) who stated that adiponectin-deficient mice have severe neointimal thickening and increased proliferation of vascular smooth muscle cells in mechanically injured arteries that can be attenuated by adenovirus-mediated adiponectin administration (Matsuda *et al*., 2002). Our findings show that the levels of adiponectin are correlated positively and negatively with the values of HDL-C and LDL-C values, respectively, in CAD group which is in agreement with the results obtained by Yutaka *et al*. (2011)**.**

Adiponectin suppresses lipid accumulation in macrophages, resulting in markedly decreased uptake of oxidized LDL and inhibition of foam cell formation which provides vasculoprotection through improvement of lipid metabolism (Ouchi *et al*., 2001), which is supporting the results obtained in the present study. The group of patients showed increase in the levels of total cholesterol, LDL-C and triacylglycerols, while, the levels of HDL-C is decreased with the decrease in the levels of adiponectin. The mechanism by which adiponectin influences lipid metabolism suggests that the positive effects of adiponectin on HDL levels which might result from its significant positive relationship with lipoprotein lipase activity. Furthermore, the discussion about the mechanism of adiponectin in atherosclerosis is inappropriate because of a lack of direct data regarding this issue. Nevertheless, these reported findings, with the present results, indicate that lower levels of adiponectin may provide certain information for predicting CAD (Yutaka *et al*., 2011)**.**

Leptin is a 26 kDa (Von *et al*., 2004), almost exclusively secreted by white and brown adipocytes (Buyse *et al*., 2001). Its expression and secretion are also regulated by a variety of other factors; for example, leptin is increased by insulin, glucocorticoids, TNF-α, and estrogen (Ouchi *et al*., 2001). Under normal conditions; leptin contributes to blood pressure homeostasis by its vasorelaxing and vasocontractile effects (Lembo *et al*., 2000). While the contractile effect of leptin is attributed to sympathetic nervous system activation (Frühbeck, 1999). Various mechanisms seem to be responsible for leptin-induced vasorelaxation. This latter effect can be

endothelium-dependent, either through the release of NO (Vecchione *et al*., 2002) or by other mechanisms (Matsuda *et al*., 2003). The vascular effects in an isolated preparation are independent of any neutrally mediated actions of leptin. They are consistent with several previous researches demonstrating leptin-induced vasodilatation of coronary artery in humans and activation of endothelial nitric oxide production in human aortic endothelial cells (Matsuda *et al*., 2003).

The administration of leptin may increase oxidative stress *in vitro* cultured human endothelial cells (Bouloumie *et al*., 2002). The increase in oxidative stress may interact with nitric oxide to form peroxy nitrite and thereby, decrease the bioavailability of nitric oxide, which is associated with an impairment of endothelium-dependent vasodilatation (Cooke and Oka, 2002).

Leptin stimulates synthesis of endothelin-1, a potent vasoconstrictor and mitogen (Quehenberger *et al*., 2002). Also, under effect of leptin, there is increase in the secretion of lipoprotein lipase enzyme in macrophages (Maingrette and Renier, 2003), and accumulation of cholesterol esters in the foam cells especially at high plasma glucose concentration (O' Rourke *et al*., 2001). There is a positive correlation between leptin and plasma concentration of fibrinogen and von Willebrand factor (Thogersen *et al*., 2004) and leptin promotes ADP-platelet aggregation (Corsonello *et al*., 2003**)**.

Leptin may also activate adult human progenitor cells and promote angiogenesis (Wolk *et al*., 2005), protect macrophages from cholesterol overload (O' Rourke *et al*., 2002). The apparent discrepancy between the protective actions of leptin and its association with impaired cardiovascular outcome in the epidemiological studies can be explained by: first, the broad spectrum actions of leptin on the cardiovascular system; second, dose dependent effects of leptin; and third, the concept of selective leptin resistance (Wolk and Somers, 2006).

In the present study, the mean value of serum leptin levels of CAD group were higher when compared to the control group and inversely correlated to the levels of serum adiponectin and correlated positively with the severity of CAD. Our findings are in agreement with the reported results of Yutaka *et al*. (2011) and Wolk *et al*. (2004), also, leptin levels show positive insignificant correlations with values of HDL-C and LDL-C. However, other investigators emphasized a potential protective role in CAD (Matsuda *et al*., 2003, Couillard *et al*., 1998**,** Piemonti *et al*., 2003).

From the results of Receiver Operating Characteristic (ROC) curve for the studied parameters, it is shown that leptin and adiponectin have the same sensitivity (100%) as biomarkers for CAD, also, leptin is more specific than adiponectin. A previous report showed that leptin levels were the most sensitive marker for predicting the accumulation cardiovascular risk factors in the general population of elementary school children (Yoshinaga *et al*., 2008). Nakatani *et al*. (2008), reported that serum leptin was a useful biomarker of metabolic abnormalities than high molecular weight adiponectin in general male adolescents.

5. Conclusion

Serum leptin and adiponectin are biomarkers for and correlated to CAD not only in the role they might play in the pathogenesis of the disease but also in their severity and leptin is a more specific biomarker than adiponectin.

The limitation to the present study is the relatively small patients' number included in the study.

The future plan will be directed towards leptin receptor gene polymorphisms and their effects on the circulating levels of leptin and the signaling capacity of leptin.

Acknowledgement

Special thanks go to Mrs. Naghum Al S'oub**,** a computer engineer, for her assistance in the statistical analyses of the obtained results of this study.

References

Adamczak M, Wiecek A, Funahashi T, Chudek J, Kokot F and Matsuzawa Y. 2003. Decreased plasma adiponectin concentration in patients with essential hypertension. *Am J Hypertens*., **16**:72–75.

Antoniades C, Antonopoulos A S, Tousoulis D and Stefanadis C. 2009. Adiponectin: from obesity to cardiovascular disease. *Obes Rev*., **10**:269–279.

Asferg C, Møgelvang R, Flyvbjerg A, Frystyk J, Jensen J S, Marott J L, Appleyard M, Jensen GB and Jeppesen J. 2010. Leptin, not adiponectin, predicts hypertension in the Copenhagen City Heart Study. *Am J Hypertens*., **23**:327– 333.

Beltowski J. 2006. Leptin and atherosclerosis. *Atherosclerosis*, **189**: 47-60.

Bjursell M, Ahnmark A, Bohlooly-Y M, William-Olsson L, Rhedin M, Peng X P, Plog K, Gerdin A K, Arnerup G, Elmgren A, Berg A L, Oscarsson J and Linden D. 2007. Opposing effects of adiponectin receptors 1 and 2 on energy metabolism. *Diabetes*, **56**:583-593.

Bouloumie A, Marumo T, Lafontan M and Busse R. 1999. Leptin induces oxidative stress in human endothelial cells. *FASEB J.*, **13**: 1231-1238.

Brubeck G. 2006. Intracellular signaling pathways activated by leptin. *Biochem J.*, **393**:7–20.

Buyse M, Viengchareun S, Bado A and Lombès M. 2001. Insulin and glucocorticoids differentially regulate leptin transcription and secretion in brown adipocytes. *FASEB J*., **15**:1357–1366.

Cheng K K Y, Lam K S L, Wang Y, Huang Y, Carling D, Wu D, Wong C and Xu A. 2007. Adiponectin-induced endothelial nitric oxide synthase activation and nitric oxide production are mediated by APPL1 in endothelial cells. *Diabetes*, **56(5)**:1387–1394.

Cooke JP and Oka RK. 2002. Does leptin cause vascular disease? *Circulation*, **106**: 1904-1905.

Corsonello A, Perticone F, Malara A, De Domenico D, Loddo S, Bumei M, Lentile R And Corica F. 2003. Leptin-dependent platelet aggregation in healthy, overweight and obese subjects. *Int J Obes Relat Metab Disord*., **27**:566-573.

Couillard C, Lamarche B, Mauriege P, Cantin B and Dagenais G R. 1998. Ischemic heart disease in men. Prospective results from Quebec Cardiovascular Study. *Diabetes Care***,** **21**:782-786.

Eguchi M, Liu Y, Shin E J and Sweeney G. 2008. Leptin protects H9c2 rat cardiomyocytes from H_2O_2-induced apoptosis. *FEBS J*., **275**:3136–3144.

Engvall E, Jonsson K and Perlman P. 1971. Enzyme- linked immunosorbent assay. II. Quantitative assay of protein antigen, immunoglobulin G, by means of enzyme-labeled antigen and antibody-coated tubes. *BiochimBiophys Acta* **251**:427-434.

Fésüs G, Dubrovska G, Gorzelniak K, Kluge R, Huang Y, Luft F C and Gollasch M. 2007. Adiponectin is a novel humoral vasodilator. *Cardiovasc Res*., **75**:719–727.

Finucane M F, Luan J, Wareham N J, Sharp S J, O'Rahilly S, Balkau B, Flyvbjerg A, Walker M, Højlund K and Nolan J J. 2009. Correlation of the leptin: adiponectin ratio with measures of insulin resistance in non-diabetic individuals. *Diabetologia* **52(11)**:2345–2349.

Friedewald W T, Levy R and Fredrickson D S. 1972. Estimation of the concentration of low density lipoprotein cholesterol without use of the preparative ultracentrifuge. *Clin Chem*., **18**:499-502.

Frühbeck G. 1999. Pivotal role of nitric oxide in the control of blood pressure after leptin administration. *Diabetes* **48**:903–908.

Gordon T, Castelli W P, Hjortland M C, Kannel W B and Dawber T R. 1977. High density lipoprotein as a protective factor against coronary heart disease. The Framingham Study. *Am J Med*., 1977; **62(5):707**-14.

Greenstein A S, Khavandi K, Withers S B, Sonoyama K, Clancy O, Jeziorska M, Laing I, Yates A P, Pemberton P W, Malik R A and Heagerty A M. 2009. Local inflammation and hypoxia abolish the protective anticontractile properties of perivascular fat in obese patients. *Circulation* **119**:1661–1670.

Hara K, Yamauchi T, Imai Y, Manabe I, Nagai R and Kadowaki T. 2007. Reduced adiponectin level is associated with severity of coronary artery disease. *Int Heart J*., **48**:149-53.

Hong S J, Park C G, Seo H S, Oh D J and Ro Y M. 2004. Associations among plasma adiponectin, hypertension, left ventricular diastolic function and left ventricular mass index. *Blood Pressure*, **13(4)**:236–242.

Jacobs NJ and VandemarkPJ. 1960. The purification and properties of the alpha-glycerophosphate-oxidizing enzyme of *Streptococcus faecalis* 10C1. *Arch Biochem Biophys*., **88**:250-255.

Jaleel F, Jaleel A, Aftab J and Rahmann M A. 2006. Relationship between adiponectin, glycemic control and blood lipids in diabetic type 2 postmenopausal women with and without complication of ischemic heart disease. *Clin Chem Acta*, **370**:76-81.

Kubota N, Terauchi Y, Yamauchi T, Kubota T, Moroi M, Matsui J, Eto K, Yamashita T., Kamon J, Satoh H, Yano W, Forguel P, Nagai R, Kimura S, Kadowaki T and Noda T. 2002. Distribution of adiponectin causes insulin resistance and neointimal formation. *J Biol Chem.*, **277**:25863- 25866.

Lembo G, Vecchione C, Fratta L, Marino G, Trimarco V, d'Amati G and Trimarco B. 2000. Leptin induces direct vasodilation through distinct endothelial mechanisms. *Diabetes*, **49**:293–297.

Lim H S, Tayebjee M H, Tan K T, Patel J V, Macfadyen R J and Lip G Y.2005. Serum adiponectin in coronary heart disease: ethnic differences and relation to coronary artery disease severity. *Heart* **91**:1605-1606.

Lin J, Yan G T, Xue H, Hao X H, Zhang K and Wang LH. 2007. Leptin protects vital organ functions after sepsis through recovering tissue myeloperoxidase activity: an anti-inflammatory role resonating with indomethacin. *Peptides*, **28**:1553–1560.

Löhn M, Dubrovska G, Lauterbach B, Luft F C, Gollasch M and Sharma A M. 2002. Periadventitial fat releases a vascular relaxing factor. *FASEB J*., **16**:1057–1063.

Maenhaut N and Van de Voorde J. 2011. Regulation of vascular tone by adipocytes. *BMC Med*., **9**:25.

Maingrette F and Renier G. 2003. Leptin increases lipoprotein lipase secretion by macrophages: involvement of oxidative stress and protein kinase C. *Diabetes*, **52**:2121-2128.

Mariman EC and Wang P. 2010. Adipocyte extracellular matrix composition, dynamics and role in obesity. *Cell Mol Life Sci.*, **67**:1277–1292.

Matsuda M, Shimomura I, Sata M, Arita Y, Nishida M, Maeda N, KumadaM, Okamoto Y, Nagaretani H, Nishizawa H, Kishida K, Komuro R, Ouchi N, Kihara S, Nagai R, Funahashi T and Matsuzawa Y. 2002. Role of adiponectin in preventing vascular stenosis: The missing link of adipo-vascular axis. *J Biol Chem*., **277**:37487-37491.

Matsuda K, Teragawa H, Fukuda Y, Nakagawa K, Higashi Y and Chayama K. 2003.Leptin causes nitric-oxide independent coronary artery vasodilation in humans.*HypertensRes*., **6**:147–152.

Nakatani H, Hirose H, Yamamoto Y, Saito I and Itoh H. 2008. Significance of leptin and high molecular weight adiponectin in the general population of Japanese male adolescents. *Metabolism*, **57**:157-162.

Otero M, Lago R, Lago F, Casanueva F F, Diequez C, Gomez-Reino J J and Gualillo O. 2005. Leptin from fat to inflammation: old questions and new insights. *FEBS Letters* **579**:295-301.

Ouchi N, Kihara S, Arita Y, Okamoto Y, Maeda K, Kuriyama H, Hotta K, Nishida M, Takahashi M, Muraguchi M, Ohmoto Y, Nakamura T, Yamashita S, Funahashi T and Matsuzawa Y. 2000. An adipocyte-derived plasma protein, inhibits endothelial NF-κB signaling through a cAMP dependent pathway. *Circulation*, **102**:1296-1301.

Ouchi N, Kihara S, Arita Y, Nishida M, Matsuyama A, Okamoto Y, Ishigama M, kuriyama H, Kishida K, Nishizawa H, Hotta K, Muraguchi M, Ohmoto Y, Yamashita S, Funahashi T and Matsuzawa Y.2001.Adipocyte derived plasma protein,suppresses lipid accumulation and class A scavenger receptor expression in human monocyte-derived macrophages. *Circulation*, **103**:1057-1063.

Ouchi N, Kihara S, Funahashi T, Matsuzawa Y and Walsh K. 2003. Obesity, adiponectin and vascular inflammatory disease. *Curr Opinion Lipidol.*, **14(6)**:561–566.

O' Rourke L, Gronning L M, Yeaman S J and Shephard P R. 2002.Glucosedependent regulation of cholesterol ester metabolism in macrophages by insulin and leptin. *J Biol Chem*., **277**:42557-42562.

O' Rourke L, Yeaman S J and Shephard P R. 2001. Insulin and leptin actually regulate cholesterol ester metabolism in macrophages by novel signaling pathways. *Diabetes*, **50**:955-961.

Piemonti L, Calori G, Mercalli A, Lattuada G, Monti P, Garancini M P, Costantino F, Ruotolo G, Luiz L and Perseghin G. 2003. Fasting plasma leptin, tumor necrosis factor-α receptor 2 and monocyte chemoattracting protein 1concentration in a population of glucose tolerant and glucose intolerant women: impact on cardiovascular mortality. *Diabetes Care*, **26**:2883-2889.

Quehenberger P, Exner M, Sunder-Plassmann R, Ruzicka K, Bieglmayer C, Endler G, Muellner C, Speiser W and Wagner O. 2002. Leptin induces endothelin-1 in endothelial cells *in vitro*. *Circ Res.,* **90**:711-718.

Richmond W. 1973. Preparation and properties of a cholesterol oxidase from *Nocardia* sp. and its application to the enzymatic assay of total cholesterol in serum. *Clin Chem*., **19(12)**:1350-1356.

Sattar N, Wannamethee G, Sawar N, Chernova J, Lawlor D A, Kelly A, Wallace A M, Danesh J and Whincup P H. 2009. Leptin and coronary heart disease: prospective study and systematic review. *J Am Coll Cardiol*., **53**:167-75.

Selcuk H, Temizhan A, Selcuk M T, Sen T, Maden O, Tekeli S and Sasmaz A. 2008. Impact of metabolic syndrome on future cardiovascular events in patients with first acute myocardial infarction. *Coron Artery Dis.,* **19**:79-84.

Shibata R, Ouchi N, Ito M, Kihara S, Shiojima I, Pimentel D R, Kumada M, Sato K, Schiekofer S, Ohashi K, Funahashi T, Colucci W S and Walsh K. 2004. Adiponectin-mediated modulation of hypertrophic signals in the heart.*Nature Medicine,* **10(12)**:1384–1389.

Tan K C, Xu A, Chow W S, Lam M C, Ai VH, Tam S C and Lam KS. 2004. Hypoadiponectinemia is associated with impaired endothelium-dependent vasodilation. *J Clin Endocrinol Metab.*, **89**:765–769.

Thogersen A M, Soderberg S, Jansson J H, Dahlen G, Boman K, Nilsson T K, Lindahl B, Weinehall L, Stenlund H, Lundberg V, Johnson O, Ahren B and Hallmans G. 2004. Interactions between fibrinolysis, lipoproteins and leptin related to a first myocardial infarction. *Eur J Cardiovasc Prev Rehabil*., **11**:33-40.

van Dielen F M, van't Veer C, Schols A M, Soeters P B, Buurman W A and Greve J W. 2001. Increased leptin concentrations correlate with increased concentrations of inflammatory markers in morbidity obese individuals. *Int J Obes Relat Metab Disord.*, **25**:1759-66.

Vecchione C, Maffei A, Colella S, Aretini A, Poulet R, Frati G, Gentile MT, Fratta L, Trimarco V, Trimarco B and Lembo G. 2002. Leptin effect on endothelial nitric oxide is mediated through Akt-endothelial nitric oxide synthase phosphorylation pathway. *Diabetes,* **51**:168–173.

Von E M, Schneider J G, Humpert P M, Rudofsky G, Schmidt N, Barosch P, Hamann A, Morcos M, Kreuzer J, Bierhaus A, Naworth P P and Dugi K A. 2004. Decreased plasma lipoprotein lipase in hypoadiponectinemia: an association independent of systemic inflammation and insulin resistance. *Diabetes Care*, **27**:1925-1929.

Wolk R, Berger P, Lennon R J, Brilakis E S, Johnson B D and Somers V K. 2004. Plasma leptin and prognosis in patients with established coronary atherosclerosis. *J Am Coll Cardiol.,* **44**:1819-1824.

Wolk R, Deb A, Caplice N M and Somers V K. 2005. Leptin receptor and functional effects of leptin in human endothelial progenitor cells. *Atherosclerosis*, **183**:131-139.

Wolk R and Somers V K. 2006. Leptin and vascular function: Friend or foe? *Eur Heart J.*, **27**:2263-2265.

Wozniak S E, Gee L L, Wachtel M S and Frezza E E. 2009. Adipose tissue: the new endocrine organ? *Dig Dis Sci.,* **54**:1847–1856.

Xi W, Satoh H, Kase H, Suzuki K and Hattori Y. 2005. Stimulated HSP90 binding to eNOS and activation of the PI3-Akt pathway contribute to globular adiponectin-induced NO production: vasorelaxation in response to globular adiponectin. *Biochem Biophys Res Commun*., **332**:200–205.

Yoshinaga M, Sameshima K, Tanaka Y, Wada A, Hashiguchi J, Tahara H and Kono Y. 2008. Adipokines and the prediction of the accumulation of cardiovascular risk factors or the presence of metabolic syndrome in elementary school children. *Circ. J.,* **72**:1874-1878.

Yutaka K, Masae I, Shunji T, Jun T, Natsuki O and Shozo K. 2011. Association of circulating levels of leptin and adiponectin with metabolic syndrome and coronary heart disease in patients with various coronary risk factors. *Int Heart J.,* **52(1)**:17-22.

Zhou Y, Sun X, Jin L, Stringfield T, Lin L and Chen Y. 2005. Expression profiles of adiponectin receptors in mouse embryos. *Gene Expr Patterns,* **5**:711-715.

JJBS

Volume 7, Number 3, September .2014
ISSN 1995-6673
Pages 179 - 185

Jordan Journal of Biological Sciences

Fish Species Assemblages in Two Riverine Systems of Mujib Basin in Jordan and the Effects of Impoundment

Nashat A. F. Hamidan*

Royal Society for the Conservation of Nature [RSCN], Jordan; and Centre for Conservation Ecology and Environmental Science, School of Applied Sciences, Bournemouth University, Poole, BH12 5BB, United Kingdom.

Received: April 4, 2014 *Revised: May 6, 2014* *Accepted: May 15, 2014*

Abstract

Mujib and Haidan rivers were sampled in July 2011 to identify fish populations in the riverine system, and to investigate the impact caused by the existence of two dams. Fishes were sampled by electofishing gear through 14 sampling points distributed along the two rivers. The Mujib Dam lake was sampled by gillnets. A total of 2,854 fish specimen were caught during the study of which most consisted of native species but included small numbers of invasive species.
The native *Capoeta damascina* and *Oxynoemacheilus insignis* were found in both rivers but *Garra rufa* was only recorded below their confluence to the west. Four invasive species were recorded. The cichlids *Oreochromis aureus* and *Tilapia zillii* were sampled along Mujib River, and the African Catfish *Clarias gariepinus* and the Common Carp *Cyprinus carpio* were observed only in the lake of Mujib Dam but were not sampled. The construction of the two dams has led to significant changes in the habitats, water flow, and hydrology of the two rivers both upstream and downstream, and enhanced the colonisation of invasive species. There is, therefore, an urgent need to review the management of the system across all stakeholders and to continue monitoring on a regular basis.

Key words: Fish diversity, Conservation, *Oxynoemacheilus insignis*, Invasive, Impact.

1. Introduction

In arid regions, the escalating demands for water have resulted in the substantial modification of many river systems (Propst *et al.*, 2008). In conjunction with the widespread invasion of many rivers by non-native fishes, this has increased the threat of local native fish populations being extirpated and endemic fishes becoming extinct (Kingsford, 2000; Olden and Poff, 2005; Propst *et al.*, 2008).

This situation exists in most of the Jordanian water bodies because Jordan is considered the fourth world poorest country in water (Denny *et al.*, 2008). Damming became a growing activity in the past decades, and major rivers in the country were impounded to allocate water for domestic use. Two of these major rivers are the Mujib and Haidan, both located within the Mujib Basin [Centre coordinates: E 36° 1' 35" N 31° 9' 52"] flowing from east to west before entering the Dead Sea. In 2002 and 2003, two dams were constructed on the Haidan (Waleh Dam) and Mujib (Mujib Dam) rivers, respectively, which caused a concern for the wellbeing and long term survival of their native fish populations. The two dams were constructed to provide a regular and more sustained supply of water year round to charge the aquifer, for drinking and agricultural purposes.

In 2003 when the reservoir was filled for the first time, fish sampling by the author revealed large numbers of introduced cichlids. Seven years later, analyses of water samples showed evidence of contamination by heavy metals (Manasreh *et al.*, 2010), after which fish monitoring was carried out by the Royal Society for the Conservation of Nature (RSCN), to assess the impact caused by the dams using the native fish populations as an indicator of impact level.

Although there are few recent studies on the fresh water fish of Jordan (e.g. Hamidan and Mir, 2003; Hamidan, 2004), none of them addressed the Mujib Basin in particular except for the tentative work jointly performed by RSCN and the Limnology Department in the University of Vienna in 2001, and the Length-weight relationships assessment of the three native species in the basin (Hamidan and Britton, 2012). Details of the fish fauna of Jordan are largely confined to taxonomic revisions provided by Krupp and Schneider (1989) all of which were completed before the above rivers were impounded. The aim of the current study is to establish a baseline data on fish diversity in the two river systems, and to identify the existing and potential threats to their survival posed by changes in water flow and impoundment.

* Corresponding author. e-mail: nashat.hamidan@rscn.org.jo.

2. Materials and Methods

2.1. Study Area

The Mujib basin (Figure 1) covers an area of 6,600 km^2 and consists of two major sub-catchments, the Wadi Mujib catchment (4,500 km^2), and the Wadi Wala (or Haidan) catchment (2,100 km^2). The rivers' beds are covered in soft sediments derived from windblown, while, in the downstream area, the slopes are steep due to flush flood erosion and the comparatively more rainfall occurrence (Al-assa'd and Abdulla, 2010). Perennial flow only occurs in downstream reaches where elevations are (- 400 m) lower than the mean sea level. The elevation in Mujib basin ranges from about 950 m a.m.s.l. southern of Karak city to an approximate of 400 m below sea level at the outlet of Wadi Mujib. The two rivers were divided into three sections for the purpose of sampling:

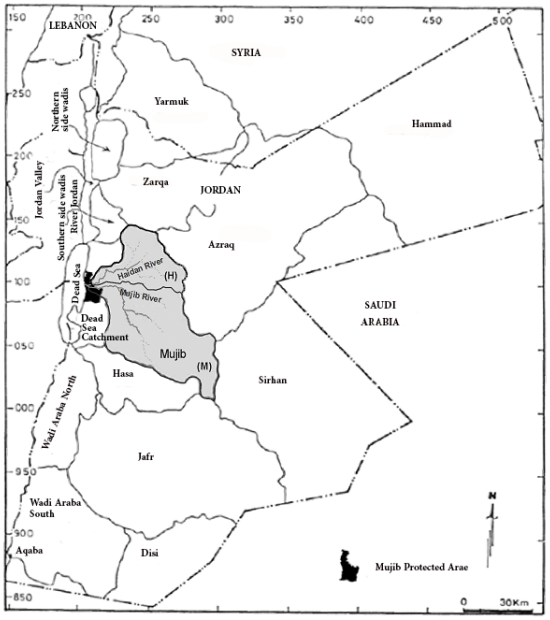

Figure 1. Location of Mujib basin in Jordan according to JICA/WAJ (Japan International Cooperation Agency/Water Authority of Jordan) (1987) Hydrologeological and water use study of the Mujib watershed, appendix (I) Final report. Amman, Jordan. Mujib Protected Area's location was added to the original map. Adapted by the author: Mujib Basin is showing the two sub-catchments: Haidan sub-catchment (H) and Mujib sub-catchment (M).

Section 1: Mujib River: This section starts from the riverhead 20 km east of the dam at Um Al-Rasas (E 35˚53'46" N 31˚23'59"), down to the confluence point locally known as *Malagi* with Haidan River passing through the Mujib dam. Two main wadies drain into the Mujib Dam locally known as Wadi Al-Sawalqah and Wadi Nkhailah where the southern wadi Nkhailah is completely dry, unlike the running northern wadi Al-Sawalqah. The water within the Mujib River is shallow and there are no deep water bodies. The water current ranges between 0.6-0.8 m/s and varied according to the area of water spreading. Most of the river is heavily vegetated with reeds. The river substrate consists of small to medium sized rocks.

In 2010, after the establishment of the dam, Manasrah *et al.* investigated the contamination of water and sediment in Mujib Dam by heavy metals. Manasrah revealed that the sediments are polluted with Cadmium (Cd), relatively contaminated with Nickel (Ni), and Zink (Zn), and uncontaminated with respect to Magnesium (Mn), Led (Pb), and Cupper (Cu).

Section 2: Haidan River: The area is located in upper Haidan River west to the Walah Dam down to the confluence point. Most of the upper wadi is dry, especially the area east to the dam; water appeared west of Walah Dam (due to the recharging process the dame is applying on the aquifer). The water is being utilised for agriculture where local farms exist along this section. Continuing west toward the Dead Sea, water disappeared and the whole wadi became dry through the Mujib Protected Area deep in a basalt canyon until reaching a 45 meter height waterfall, water starts to flow with the aid of side springs supplying the river.

Water depth varies from few centimeters of flowing water to 4 metre deep ponds within the canyon areas. Current speed varies from 0 m/s up to 0.5 m/s. For agricultural purposes, water is being pumped from the river or from the dam pond to irrigate the high water consuming corps. The substrate consists of small sized rock beds to larger sized rocks within the canyon. A few areas downstream are heavily vegetated with reeds that cover the whole river area.

Section 3: Mujib and Haidan: This is the last section where both Mujib and Haidan Rivers meet then drain to the Dead Sea along 3.4 km distance through Mujib canyon, where 12 metres height water fall occurs after 1.2 km to the west of the Mujib canyon entrance. Water is shallow, and the current speed is the highest where it reaches 1 m/s in the canyon. Vegetation is minimal except for the downstream where the canyon is becoming wide (maximum of 8 metres) and vegetation of reeds, Oleander and *Typha* are the most dominant plant species. Before reaching the Dead Sea, water is converted to water treatment station, treated, and pumped for human consumption leaving behind a minimum amount of water drained to the Dead Sea.

2.2. Fish Sampling

Fish were sampled in 14 sites along the basin during July 2011. The sampling location was distributed to six sites in Mujib River section, three sites in Haidan River system, and five sites in Mujib-Haidan system downstream of the confluence point (Figure 2).

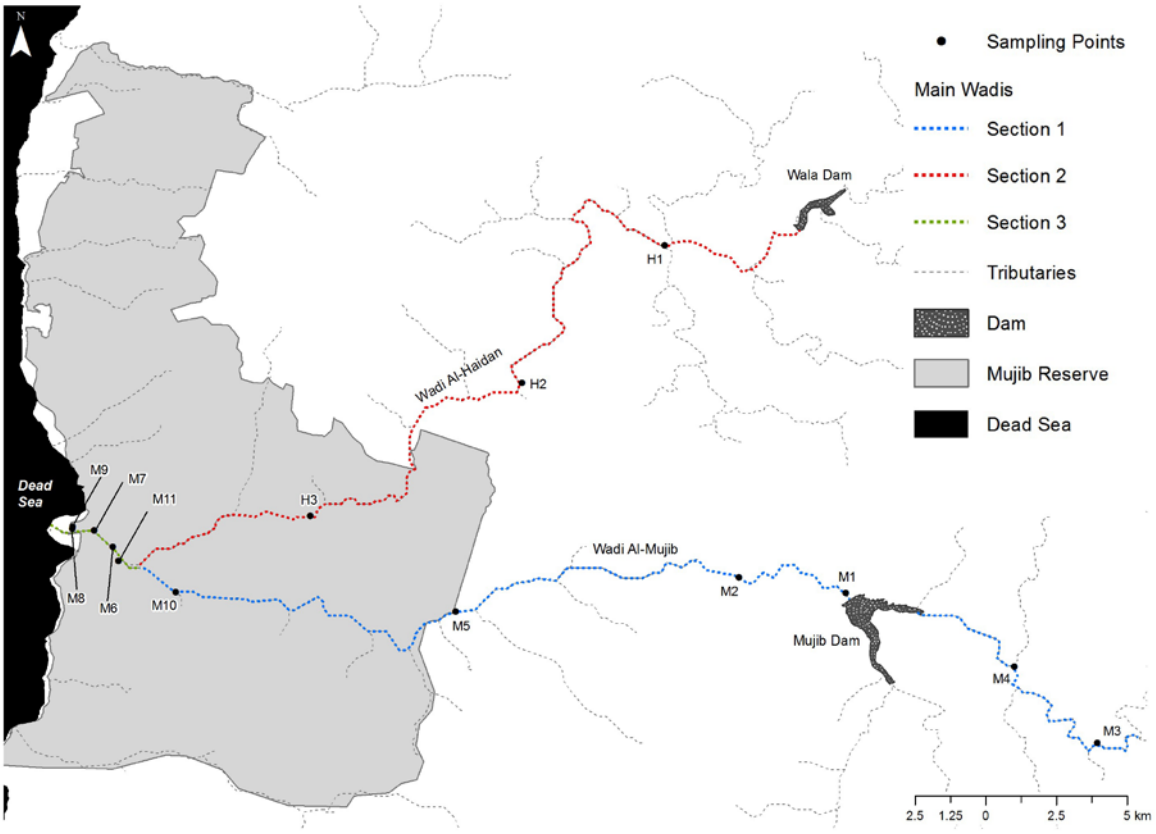

Figure 2. The study area where water was available along the two rivers, including the sampling points.

The sampling sites were designed to reflect a representative sample of habitats along each section. Sampling is comprised by battery-powered, back-mounted electric fishing gear, while gill nets of mesh sizes up to 22 mm was only used in the lake of the Mujib Dam. Once captured, fish were identified to species level, measured (standard length, SL, nearest 1 mm), weighed (to 0.01 g), and immediately released alive at the sampling site. Data on length-weight relationship were reported by Hamidan and Britton (2012). Fish standard length were categorised into 1 cm groups, and plotted against number of fish individuals in the same size group.

3. Results

A total of 2,854 specimens representing seven species were caught during the study. These species include *Capoeta damascina* (92% of total catch), *Oxynoemacheilus insignis* (6.1 %), *Garra rufa* (1.15 %), and the alien *Oreochromis aureus* (3.5%) and *Tilapia zillii* (7.02%). The African Sharptooth Catfish *Clarias gariepinus* and the Common Carp *Cyprinus carpio* were both observed in the Mujib dam lake.

Mujib River section: Four species were identified in this section including two native species *C. damascina* and *O. insignis* coexisting with two introduced cichlids *O. aureus* and *T. zillii*. The population structure of *C. damascina* was dominated by Young of the Year (YOY) fish from 10-40 mm length class, while few large specimens of length 230 mm were presented (Figure 3-A). As for *O. insignis* the population structure was dominated by medium sized fish class of 50-70 mm length (Figure 3-B).

Haidan River: large numbers of fishes were found trapped in isolated ponds as a result of flow discontinuity, while others were found dead in other ponds that were dried up completely (Figure 4). Two native species were found to inhabit this river, *C. damascina* and *O. insignis*. No introduced fish were sampled, although local fishermen assured the existence of cichlids in the river. Population structure for the *C. damascina* showed dominance of (YOY) fish of 30-40 mm length class. (Figure 3-C), while for *O. insignis* the population structure showed the dominance of YOY from the 3-4 mm length class (Figure 3-D).

Mujib-Haidan: the native *Garra rufa* appeared after the high waterfall in addition to the two native species *C. damascina* and *O. insignis.* One specimen of *T. zillii* was found close to the river mouth at point M7. *Capoeta damascina* showed a population structure dominated by YOY from 30-50 mm, few individuals from the length class 210-220 were frequently sampled (Figure 3-E). *Oxynoemacheilus insignis* population structure was found to be dominated by larger seized individuals, with few YOY presented, while *G. rufa* population structure showed a population dominated by YOY from the size class of 20-30 mm (Figure 3-F), and (Figure 3-G), respectively.

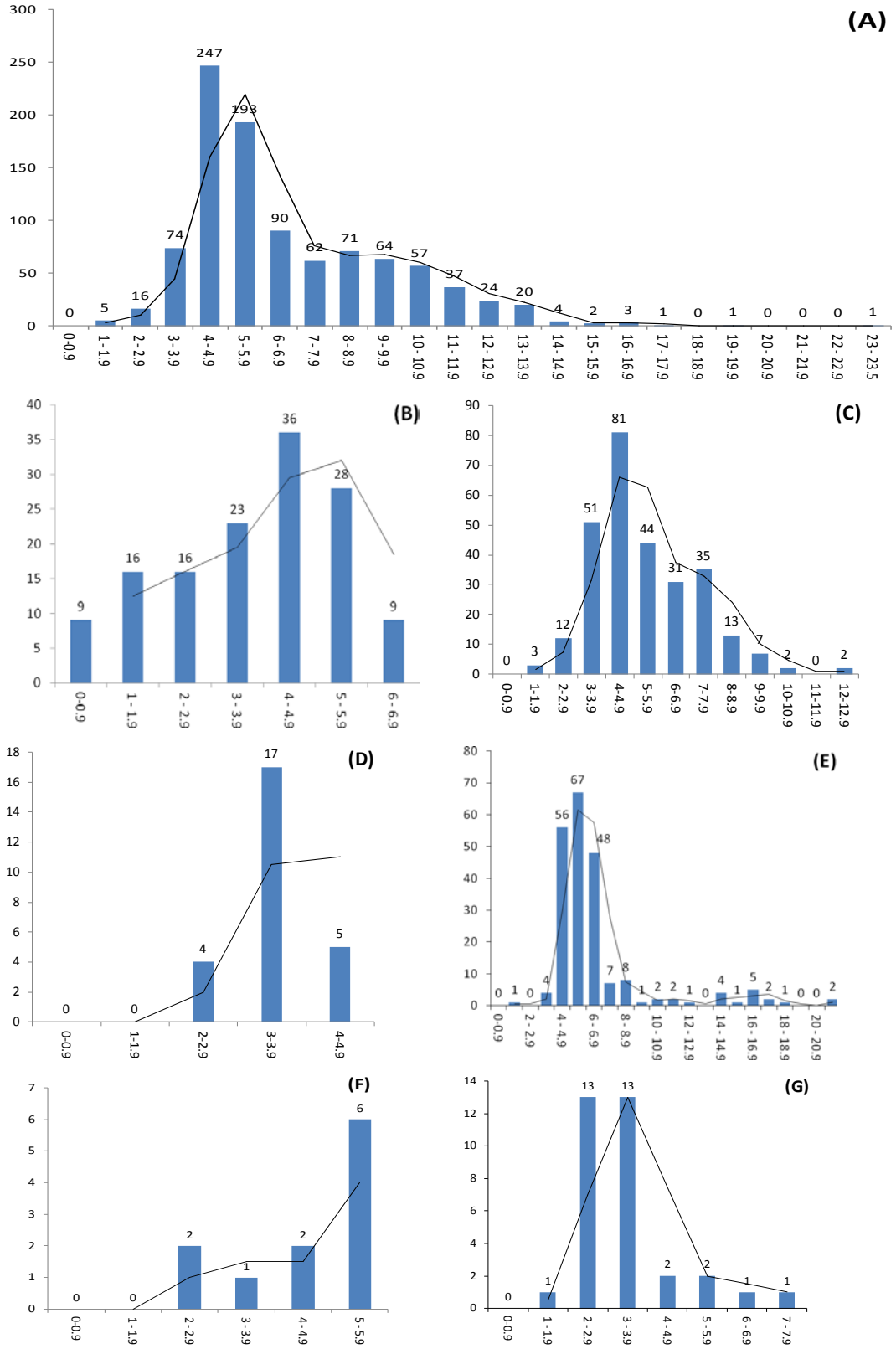

Figure 3. Population structures of native fishes in each section of the study area. Standard length is plotted on the X-axis in centimetre, and the number of individuals in each category at Y-axis. **(A)** Population structure of *C. damacina* in Mujib River (n=972), **(B)** Population structure of *O. insignis* in Mujib River (n=137), **(C)** Population structure of *C. damacina* in Haidan River (n=281), **(D)** Population structure of *O. insignis* in Haidan River (n=26), **(E)** Population structure of *C. damacina* in Mujib-Haidan River section (212), **(F)** Population structure of *O. insignis* in Mujib-Haidan River section (n= 11), and **(G)** Population structure of *G. rufa* in Mujib-Haidan River section (n=33).

4. Discussion

This study assures the importance of Mujib basin in hosting three populations of native species of the Jordanian ichthyofauna, including *G. rufa* at its southern most edge of distribution in Jordan, represented by an isolated population down to the Mujib River close to the Dead Sea. Mujib Dam is known for its high catch of fish in general among fishermen (Hamidan, Per. Comm., 2010). The low number of fish in the dam is probably due the recent introduction of the Catfish *Clarias gariepinus*, and the accumulative impact caused by the Common Carp, *Cyprinus carpio*, while *Clarias gariepinus* is well known for its predation of fish fry and even juveniles (Tawwab, 2005); *Cyprinus carpio* is the third most frequently introduced species in the world "known to be the keystone ecosystem engineer that altering habitats for native fish and other native aquatic species" as stored on the Global Invasive Species Database.

In Walah Dam, the system is different than Mujib, since the dam receives water from the rain water runoff; the water then recharges the aquifer, and no permanent rivers feed into the dam. As a result the dam lake is not a favourable habitat for native or introduced species. In addition, the dam lake dries regularly, and is mechanically cleaned. In addition to the limited accessibility to the dam, these factors reduce the chance of introducing invasive fish species. The extensive water pumping from the Haidan River over a long distance from Walah Dam west to the entrance of the river into the protected area clearly resulted with discontinuity of the water flow. A large number of fish was found dead in groups due to the gradual drying of their isolated water bodies where they were trapped (Figure 4). This, happening prior to the spawning season, resulted in the removal of breeding adults which will consequently cause the population declining at long run. This was not the case in Mujib River where the fish content and structure were healthier.

Figure 4. Number of native fish found dead along Haidan River because of drought.

In terms of the fish compositions in the river, the introduced cichlids start to appear in low numbers, where the numbers became lower when moving west. This is due to the nature of those cichlids since they are not well adapted to the fast running water, although they are well known for their great breeding potential and the explosive increases in population (Buntz and Manooch, 1968). The number of juveniles was very limited and restricted to the stagnant water on river banks, where this kind of water is regularly washed off in the seasonal floods and cause drifting of those cichlids down to the Dead Sea.

In Mujib-Haidan section, the water velocity starts to increase since the elevation is decreasing, and the well adapted bottom dweller *G. rufa* starts to appear after the natural barrier, represented by the Mujib Waterfall. The relative abundance of *O. insignis* was found to be lower than those of *G. rufa* and this could be linked to the fact that *G. rufa* is well adapted to fast running water due to its specialised mental desk. *Capoeta damascina* is still dominating the whole system since it is widely distributed in the basin and is being well adapted to both stagnant and running water.

The population structure of *C. damascina* showed healthy population dominated by YOY, which confirms the breeding success in the past season. *Oxynoemacheilus insignis* did not present such type of structure except in Haidan, however, for this species, in particular, there is almost nothing known about its biology (Krupp and Schneider, 1989); this is challenging the judgment on the species' population structure.

Several threats were identified during the present survey and earlier when the Mujib dam in particular was in operation. The first and most important impact causing factor is the controlling of the natural flow regime resulting in unexpected flooding.

Flow regime is an important determinant of the reproductive success of native and non-native fish species in regulated rivers (Brown and Ford, 2002). Controlling the flow enhances the growth of reed along the river, where it used to be washed out annually. Reed, in some location, is in high density covering the river and minimising the fish utilised habitats and forming a natural barrier along the river. The unexpected floods in summer due to cleaning processes in the dam causes significant removal of fish fry and YOY by washing them down to the Dead Sea, knowing that the period of May to June is the breeding season of the dominant *C. damascina* (Asadollah, 2011).

The dam lakes also promotes "invasive friendly receiving environment" since the introduced species, including one of the globally worst invaders, the Common Carp, which cannot adapt to the fast running water in Mujib, and/or the regular flooding cycle. The Lake of the dam makes such environment suitable for introduced species, and induces a 30-40 meters deep pool that is not known along the river system. Having no native species in the dam is only an indicator that those species (native) could be overstepped by the new invading species mainly the Common Carp and the Catfish. On the other hand, cichlids leaked to the river system out of the dam, but they did not succeed to establish a viable population because they are not adapted to such an environment.

The risk of the river has been invaded by more invasive species that can adapt to the running water and the flood cycle is not excluded. The potential expansion of catfish down the river is possible since this species is found in other running water bodies, like Zarqa River (Hamidan, personal observation, 2011). On the other hand, pollution will be - if not already is - transferred to the native and introduced fish in the dam, and consequentially to the river system down to the Dead Sea. Notes from the protected area staff show a high number of dead fish downstream from time to time.

Fishing is being practiced in and around the Mujib dam, where it is strictly illegal in the dam lake due to safety considerations. This fishing practice encouraged fishermen to introduce more high fish meat contents like Carp and Catfish, although the native *C. damascina* is well known to fishermen for its local name *Haffaf* and its taste, and is still targeted. As a result, productive individuals from the populations have been removed annually. Both dams and the surroundings are located in the "allow-hunting" area, where hunters used to target wildfowl species using the lead shots. In Mujib Dam, hunting is not allowed by the dam authority, and the lake of the dam is designed not to have any shoreline, where hunters cannot bring their hunts. A number of ducks was found dead in the dam after being shot, where they deteriorated, enriching the organic matter concentration in the impounded water. Furthermore, the type of shots that were used in hunting also enhanced the lead content in the lake of the dam, and this will eventually be transferred to fish and birds.

In conclusion, this survey confirmed the importance of Mujib-Haidan basin for three native species of Jordanian ichthyofauna. However, establishing the dams of Mujib and Walah has modified the natural water system, and blocked large amount of water behind. This water used to flow down to the Dead Sea without being controlled. In addition, the dam controls the annual flooding cycle that forms one of the major determinant features in this kind of river system. The dam indirectly facilitates the introduction of alien species including the globally third introduced species *C. carpio*. Currently, none of the invasive species has managed to establish viable population out of the dam lake. But potential coming threats are still possible if "fast running water" adaptive species are introduced, or if the catfish manages to escape into the river.

The major impact on the river system and the consequences of the impoundment are classified as anthropogenic factors mainly for agricultural practice, fishing, and hunting. Management of Mujib Protected Area needs to consider the integrated approach in watershed management including multi-sectorial involvement of stakeholders, while promoting both the upstream management concept, and environmental friendly and agriculturally sustainable practices.

Acknowledgement

This work was funded by Wetlands International and the Canadian International Development Agency References. The author is indebted to all team members who helped in the field work especially Ehab Eid, Omar Abed, Thabet Al-Share, Tareq Qaneer and Habes Amareen without whose kind help and support this work would have not been completed. Thanks also go to Natalia Bolad and Heba Dawood who prepared the maps for this work. Mujib Protected Area staff and the Mujib Dam staff are acknowledged for their support and hospitality during the study. Field companions, including Prof. Zuhair Amr, Loay Azam and Rami Al-Omari, all members of the Odonata survey team, are appreciated. Drs. Chris Goldspink (Manchester Metropolitan University), Dawoud Al-Eisawi (Jordan University) and Robert Britton (Bournemouth University) are all acknowledged for their comments on the manuscript.

References

Al-Assa'd T and Abdulla F. 2010. Artificial groundwater recharge to a semi-arid basin: case study of Mujib aquifer, Jordan. *Environ Earth Sci.*, **60**:845–859

Asadollah S, Soofiani N, Keivany Y and Shadkhastz M. 2011. Reproduction of Capoeta damascina (Valenciennes, 1842), a cyprinid fish in Zayandeh-Roud River, Iran. *J Applied Ichthyol.*, **27**:1061-1066.

Bagenal TB and Tesch FW. 1978. Age and growth. In: T. Begenal (Ed.), **Methods for Assessment of Fish Production in Fresh Waters,** 3rd Edn. IBP Handbook No. 3, Blackwell Science Publications, Oxford: 101- 211 – 216

Bolger T and Connolly P L. 1989. The selection of suitable indices for the measurement and analysis of fish condition. *J Fish Biol*., **34**:171-182.

Brown L and Ford T. 2002. Effects of flow on the fish communities of a regulated California River: implications for management native fishes. *River Res Applications*,**18**: 331–342.

Buntz J and Manooch CS. 1968. *Tilapia aurea* (Steindachner), a rapidly spreading exotic in south central Florida. Proc. SE Assoc. *Game Fish Comm*., **22**: 495-501

Denny E, Donnelly K, McKay R, Ponte Gand Uetake T. 2008. Sustainable Water Strategies for Jordan. *University of Michigan, Ann Arbor*.

Froses R and Pauly D. 2000 **FishBase: Concepts, Design and Data Sources**. ICLARM, Los Baños, Laguna, Philippines. 344 p.

Global Invasive Species Database, at: http://www.issg.org/database/species/search.asp?sts=sss&st=sss&fr=1&sn=common+carp&rn=&hci=-1&ei=-1&lang= EN

Hamidan N. 2004. The freshwater fish fauna of Jordan. *Denisia*, **14**: 385 – 394.

Hamidan N and Britton J R. 2013. Length-weight relationships for three fish species (*Capoeta damascina, Garra rufa, and Oxynoemacheilus insignis*) native to the Mujib Basin, Jordan. *J Appl Ichthyol*.,, **29** : 480-481.

Hamidan N and Amir S. 2003. The status of *Garra ghoronsis* in Jordan: distribution, ecology and threats. *Zool Middle East*, **30**: 49-54

Jackson P B N. 1989. Prediction of regulation effects on natural biological rhythms in south-central African freshwater fishes. *Regulated Rivers: Research and Management*, **3**: 205-220.

Kingsford RT. 2000. Ecological impacts of dams, water diversions and river management on floodplain wetlands in Australia. *Austral Ecol*.,**25**: 109-127.

Krupp F and Schneider W. 1989. The fishes of Jordan River drainage Basin and Azraq Oasis. *Fauna of Saudi Arabia*, **10**: 384-410.

Manasreh W, Hilat I and El-Hansan T. 2009. Heavy metal and anionic contamination in the water and sediments in Al-Mujib reservoir, central Jordan. *Environ Earth Sci*., **60**:613– 621

Moutopoulos DK and Stergiou KI. 2002. Length–weight and length–length relationships of fish species from the Aegean Sea (Greece). *J Appl Ichthyol.*, **18**: 200–203.

Olden JD and Poff NL. 2005. Long-term trends in native and non-native fish faunas of the American Southwest. *Animal Biodiversity and Conservation*,**28**: 75-89.

Propst DL, Gido KB and Stefferud JA. 2008. Natural flow regimes, nonnative fishes and native fish persistence in arid-land river systems. *Ecological Applications*,**18**: 1236-1252.

Tawwab M. 2005, Predation efficiency of Nile Catfish, *Clarias gariepinus* (Burchell, 1822) on Fry Nile Tilapia, *Oreochromis niloticus* (Linnaeus, 1758): Effect of prey density, predator size, feed supplementation and submerged vegetation. *Tur J Fisheries and Aquatic Sci.*, **5**: 69-74 .

JJBS

Volume 7, Number 3, September .2014
ISSN 1995-6673
Pages 187 - 194

Jordan Journal of Biological Sciences

In Vitro Activity of Novel Metronidazole Derivatives on Larval Stages of *Echinococcus granulosus*

Wafa'a T. Nasr[1], Haythem A. Saadeh[2,3], Mohammad S. Mubarak[2] and Sami K. Abdel-Hafez[1,*]

[1]*Department of Biological Sciences, Yarmouk University, Irbid, Jordan;*

[2]*Department of Chemistry, The University of Jordan, Amman, Jordan;* [3]*Department of Chemistry, United Arab Emirates University, Al Ain, Abu Dhabi, United Arab Emirates*

Received: April 22, 2014 *Revised: May 16, 2014* *Accepted: May 22, 2014*

Abstract

The effects of metronidazole (MTZ) and novel synthesized MTZ derivatives on *in vitro* cultured *Echinococcus granulosus* protoscoleces (PSCs), 30 day old segmentation stage and hydatid cysts (HC) developing secondarily in BALB/c mice were compared to those caused upon treatment with comparable doses of albandazole (ABZ) and mebendazole (MBZ) drugs. The highest protoscolicidal action resulted from the use of a non-schiff based MTZ derivative (MTZ-w: 4-[2-(2-methyl-5-nitro-1H-imidazol-1-yl) ethyoxy] benzeyldehyde). Incubation of PSCs with MTZ-w concentrations of 25, 12.5 and 6.25 µg/ml resulted in significantly higher mortality rates than those caused by ABZ or MBZ at all periods post incubation. Total mortality of PSCs always occurred one day earlier using MTZ-w. Moreover, incubation of PSCs with MTZ-w at 6.25 µg/ml concentration resulted in greater mortality of PSCs than that caused by ABZ at 25ug/ml concentration. Three other MTZ derivatives showed similar *in vitro* effects on PSCs to those caused by ABZ or MTZ. Light microscopy revealed that changes in PSCs exposed to MTZ derivatives and ABZ reflected their relative actions in targeting scolex hooks, suckers and tegument. MTZ-w and ABZ caused rupture of hooks, deformation in suckers and disintegration in tegument of both PSCs and *in vitro* cultured segmentation stage. Less detrimental changes occurred upon the exposure to other MTZ derivatives. Exposure of HC to MTZ-w and ABZ caused regression in their size, damage in germinal membrane, fragmentation of underlining tissue, and scaling of laminated membrane. MTZ-w warrants further assessment as a potential chemotherapeutic drug against cystic echinococcosis in both animals and humans.

Keywords: *Echinococcus granulosus*, Protoscolex, Hydatid cysts, Albendazole, Mebendazole, Metronidazole, Metronidazole derivatives.

1. Introduction

Cystic echinococcosis (CE) or unilocular hydatidosis is a cosmopolitan cyclozoonotic helminthic disease of livestock and humans with great public health and economic effects in various continents. While it is currently spreading into new developing countries and increasing in prevalence, CE is still classified with the emerging or re-emerging neglected diseases (Moro and Schantz, 2009; McManus, 2010; Da Silva, 2010).

The disease is caused by the ingestion of embryonated eggs of the tiny dog tapeworm *Echinococcus granulosus* (Eucestoda, Platyhelminthes) whose adult stage inhabits the small intestine of dogs, or any of the canid family as the main definitive host. In livestock and humans, unilocular hydatid cysts (HC) develop in various visceral organs – mainly liver and lungs. Each HC contains an outer a cellular laminated layer (LL) and inner cellular germinal layer (GL) that undergoes asexual reproduction resulting in huge number of protoscoleces (PSC) in a fluid filled environment. Symptoms are often caused when cysts make mechanical pressure on the surrounding tissues and by cyst rupture and aggregated secondary infection. Moreover, spillage of cyst fluid containing PSC leads to secondary hydatidosis (Eckert and Deplazes, 2004; McManus, 2010).

Current treatment of CE depends on one or a combination of the following strategies: surgery, puncture of cyst- aspiration-injection of protoscolicidal chemicals

* Corresponding author. e-mail: skhafez@yu.edu.jo;skhafez@yahoo.com.

and re-aspiration (PAIR), and chemotherapy (Eckert and Deplazes, 2004; Kern, 2006).However, none of these strategies is a conclusive treatment of human CE. Chemotherapeutic treatment of CE depends mainly on the use of benzimidazole compounds particularly albendazole (ABZ) and alternatively mebendazole, praziquantel and nitazoxanide (Hemphill and Muller, 2009). However, the non-optimal efficacy of these drugs, long periods of treatment needed, and the suffering caused to patients from serious side effects warrant careful search for alternative therapeutic approaches(Moro and Schantz, 2009; Hemphill and Muller, 2009; Vuitton, 2009). Chemotherapeutic applications based on the discovery of novel drugs for treatment of CE are thus needed (Vuitton, 2009; Ceballos *et al.,* 2009; Gavidia *et al.,* 2009). Such drugs should have selective and rapid scolicidal effects for both PSCs and HC stages with minimal local and systemic adverse effects on the host. It has been postulated that drugs which have been found to be effective against other eukaryotic protozoal and helminthic parasites and/or cancer cell lines are primary candidate choices for testing against CE (Hemphill and Muller, 2009). Metronidazole (MTZ) and many of its newly synthesized derivatives match these properties, and also have been found to inhibit certain cancer cell lines and the growth of cultured *Giardia intestinalis* and *Entamoeba histolytica* (Abu Shaireh *et al.,* 2009; Saadeh *et al.,* 2010; 2011).

This study was designed to investigate the effects of MTZ and many of its newly synthesized Schiff-based and non-Schiff based derivatives against freshly prepared PSC and *in vitro* cultured stages of *E. granulosus*. Moreover, the ultrastructural effects of the most effective protoscolicidal compounds on secondary HC developing in mice were explored.

2. Materials and Methods

2.1. Parasites

PSCs were isolated from the livers of infected indigenous sheep slaughtered at abattoirs in Jordan as described previously (Hijjawi *et al.*, 1992). All steps were done under sterile conditions using a vertical laminar flow hood (Flow lab, Irvine, Scotland, UK). Infected sheep offal was washed using soap, and well defined cysts were painted three times with a solution of 1% iodine in 95% ethanol. The hydatid fluid (HF) containing PSCs was aspirated using 20 ml sterile syringe fitted with a 19g needle. PSCs were collected aseptically from the HF of fertile cyst or by scrapping the GL of fertile cysts. The viability of fertile cysts was measured as a relative number of live PSC to total number of them. At least three samples were counted to determine PSC viability with a minimum of 100 PSC/ sample. Discrimination between live and dead PSC was made using methylene blue dye as a vital stain (Gold, 1997, Liu *et al.,* 2013). Only HC with at least 80% viability and free from bacterial contamination were used. Live PSC were separated from dead ones that were digested out using trypsin suspension solution prepared in phosphate buffer saline (PBS) in a 1:10 ratio. Trypsin treatment was made in water bath at 37°C with gentle shaking (60 cycles per minutes) for 30 min. *In vitro* culturing of freshly prepared PSCs and subsequent developing stages was carried out as described by Hijjawi *et al.* (1997). All experiments were carried out in 24 well culture plates. RPMI 1640 containing 20% (v/v) fetal calf serum (Invitrogen, Grand Island, New York, USA), 0.45% (w/v) yeast extract, 0.4% (w/v) glucose, penicillin/streptomycin suspension containing 400 IU penicillin and 400 µg/ml streptomycin (Flow Lab, Irvine, Scotland) and amphotericin B suspension containing 400 µg (Hyclone Labs, Thermo Scientific, Logan, Utah, USA) was used as the standard culture medium (SCM).

To prepare the first segmentation stage of *E. granulosus*, PSCs were cultured in RPMI-1640 SCM for 30 days in 160 ml culture flasks. The culture medium was changed weekly. These 30 day old cultured stages reached the first segmentation stage (S5 stage using Smyth's designation) (Smyth, 1967).

Secondary hydatidosis was developed in five BALB/c female mice which were injected subcutaneously with 1000 freshly isolated PSCs prepared in 1ml PBS (pH 7.2) when mice were two weeks old (Kakru *et al.,* 2008). After four months, mice were killed by cervical dislocation and developing HCs were dissected out from subcutaneous tissue and maintained in RPMI-1640 medium. Clumped cysts were separated individually and washed three times in PBS (pH 7.2) containing 400 IU/ml penicillin and 400 µg/ml streptomycin before being exposed to standard drugs and chemical compounds (see below).

2.2. Drugs and Chemical Compounds

Drugs and chemical compounds (Figure 1) that were tested for their efficacy against cultured PSCs and metacestode stages include Albendazole (ABZ) [Methyl 5-propylthio-2-benzimidazolecarbamate] (Satish Joshi, Kikma Pharmaceuticals, Mumbai, India) which was used as a positive control drug of choice for the treatment of CE, Mebendazole (MBZ) [5-benzoyl-1H-benzimidazol-2-yl] (Satish Joshi, Kikma Pharmaceuticals, Mumbai, India) which was used as another positive control commercial drug, Metronidazole (MTZ) [1-(2-Hydroxy-1-ethyl)-2-methyl-5-nitroimidazole] (Acros Organics, New Jersey, USA) and the following novel MTZ derivatives that were prepared, purified, and characterized previously (Abu Shaireh *et al.,* 2009; Saadeh *et al.,* 2010; 2011).

(MTZ-a):(4-Fluoro-benzylidene)-[2-(2-methyl-5-nitro-imidazol-1-yl)-ethyl]-amine,
(MTZ-b:((4-Methyl-benzylidene)-[2-(2-methyl-5-nitro-imidazol-1-yl)-ethyl]-amine,
(MTZ-c): (4-Methoxy-benzylidene)-[2-(2-methyl-5-nitro-imidazol-1-yl)-ethyl]-amine,
(MTZ-d):(4-Nitro-benzylidene)-[2-(2-methyl-5-nitro-imidazol-1-yl)-ethyl]-amine
(MTZ-e):2-{[2-(2-Methyl-5-nitro-imidazol-1-yl)-ethylimino]-methyl}-phenol,
(MTZ-f):4-Chloro-2-{[2-(2-methyl-5-nitro-imidazol-1-yl)-ethylimi-no]-methyl} phenol,
(MTZ-g):(2-Chloro-benzylidene)-[2-(2-methyl-5-nitro-imidazol-1-yl)-ethyl]-amine,
(MTZ-h):[2-(2-Methyl-5-nitro-imidazol-1-yl)-ethyl]-thiophen-2-yl methylene-amine,
(MTZ-w):4-[2-(2-methyl-5-nitro-1H-imidazol-1-yl) ethyoxy] benzeyldehyde.

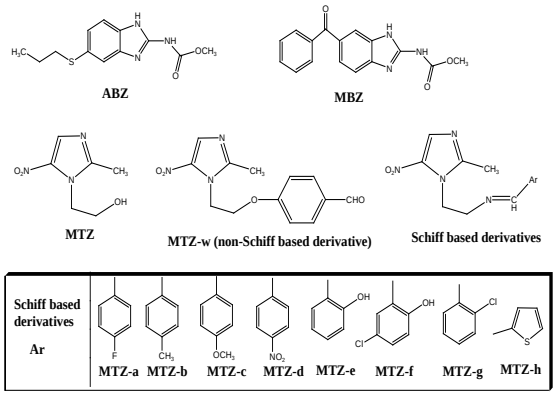

Figure 1. Schematic representation of the chemical structure of: ABZ: Albendazole; MBZ; Mebendazole; MTZ: Metronidazole MTZ derivative compounds (MTZ-w, a non-Schiff based derivative and MTZ-a - MTZ-h Schiff based derivatives).

2.3. Experimental Assays

2.3.1. Exposure of in vitro cultured PSCs and meta cestode stages to standard drugs and MTZ derivatives

For each drug or MTZ derivative, a stock of 200 µg/ml was dissolved in 50% DMSO solution that was initially prepared in sterile double distilled H2O. The stock compounds were sterilized using 0.20 µm filters (Whatman, NJ, USA). Each well received 2 ml of SCM containing 25, 12.5 or 6.25 µg/ml of each compound that was freshly prepared from the original standard drugs or chemical compound stocks. Some 2000 PSCs were added to each culture well and incubated for 13 days at 37 °C with 95% humidity and 5% CO2 in air. The pH of the cultures was adjusted to pH 7.4 at the beginning of the experiment. The PSC viability was tested daily by counting viable and dead PSC in a minimum of 50 PSC taken from each well and various drug and derivatives treatments were carried out in triplicate wells for each concentration used. Viability of PSCs was determined using methylene blue vital stain. The stain penetrates dead PSCs which appeared intensely stained with the dye. ABZ was used as positive control in addition to three negative controls: SCM, 50% DMSO solution and a combination of SCM and 50% DMSO solution in a 1:1 ratio.

2.3.2. Exposure of in Vitro Cultured First Segmentation Metacestode Stage to Standard Drugs and MTZ Derivatives

The first segmentation stages (S5 metacestodes) that were prepared upon *in vitro* culturing of PSCs for 30 days were exposed to the same drug concentrations following a similar protocol to that described above for freshly prepared PSCs. The morphological, anatomical and parasiticidal effects on the developing stages was followed for 14 days.

2.3.3. Exposure of secondarily developed HC to MTZ-w and ABZ

Five secondarily developing HCs that were isolated from BALB/c mice as described above were incubated with SCM containing 25 µg/ml of MTZ-w for 21 days. Other HCs were incubated with a comparable concentration of ABZ that was used as a standard positive control drug, while others were incubated with a solution made of SCM and 50% DMSO in a 1:1 ratio and used as negative control.

2.4. Microscopic Examination

2.4.1. Light microscopy

Morphological and tegumentary changes in cultured PSCs subjected to various drug and MTZ derivatives were studied under light microscopy using aceto-carmine staining protocol (Meyer and Olsen, 1980). Briefly, PSCs or cultured larval stages were fixed in 10% formalin solution for at least 48 hs. The specimens were washed three times with distilled water before being dehydrated through an ascending ethanol series (35%, 50% and 70%) for 30 min each. Then, 70% ethanol was replaced by Semichon'saceto carmine stain for 30 min. Samples were washed with 70% ethanol for few seconds. Excessive staining was avoided by placing specimens in 70% ethanol containing 2-4 drops of HCL for few minutes until they differentiated well. Subsequently, specimens were washed quickly with 70% ethanol for few seconds before they were transferred to 70% ethanol with 2 drops of NaHCO3 solution and kept there for 30-60 min. Further dehydration was made through further ascending ethanol series (85%, 95% and 100%) for 15 min each. After further dehydration with 100% ethanol, samples were transported to glass vials containing xylene and kept there for at least 15 min before being mounted on a glass slide supported with one drop of Canada balsam, covered and dried.

A minimum of 25 stained specimens were examined to determine the microscopic effects of compounds on treated PSCs and *in vitro* cultured metacestode stages.

2.4.2. Scanning electron microscopy

Metacestodes that were exposed to standard positive control drugs or MTZ derivatives were picked from culture medium and washed three times with sodium cacodylate buffer (SCB) (pH 7.2) for 5 min each. Then, they were fixed in 2.5% glutaraldehyde in 0.1M SCB for four h. After washing three times with 0.1 M SCB for 5 min each, specimens were post fixed in 1% osmium tetroxide (OsO4) prepared in the same buffer for two hours. The cysts were washed further three times with SCB for 5 min each. Next, they were dehydrated through an ascending ethanol series (30, 50, 70, 90, and 100%) for 20 min each. Finally, the specimens were dried using Balzers critical point drier 0301 (Wanner *et al.*, 2005) and sputter coated with gold on stubs. The specimens were studied and photographed using Zeiss scanning electron microscope at 20 KV on a rotator.

3. Results

3.1. In vitro effects of MTZ derivatives on fresh PSCs

Among all MTZ derivatives tested, the highest protoscolicidal action resulted from the use of MTZ-w compound in which the mortality rates were consistently higher than comparable ABZ or MBZ drug concentrations (25, 12.5 and 6.25 µg/ml) throughout the periods of post-incubation with these compounds (Figures 2-4).

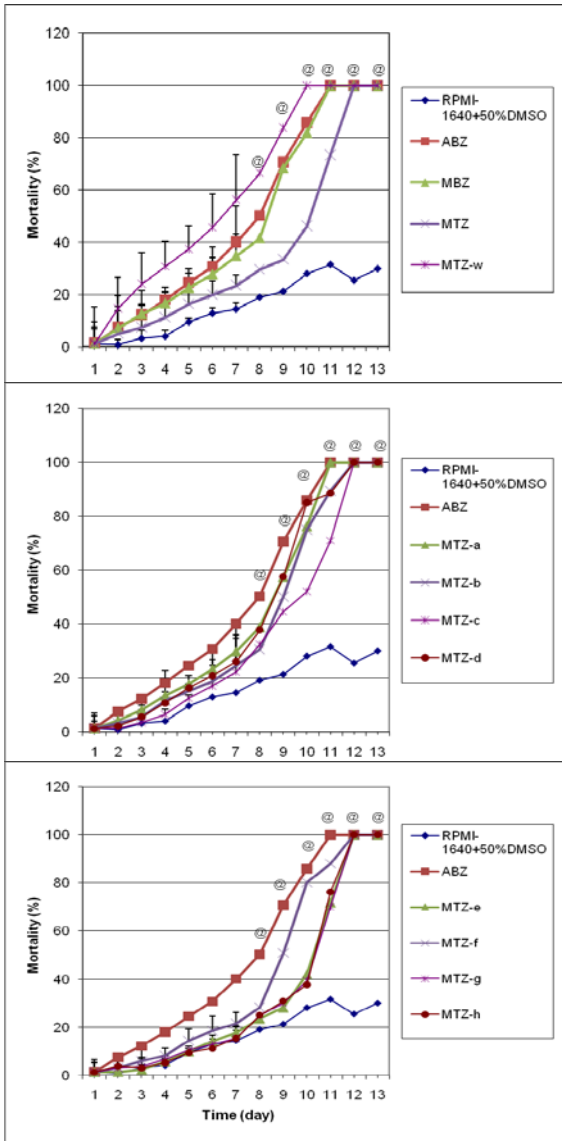

Figure 2. Mean percent mortality rates of freshly cultured *E. granulosus* PSCs with RPMI-1640 and treated with 25 µg/ml ABZ, MBZ, MTZ or its derivatives. " @: standard deviation was not placed because the values represent only one or two observations".

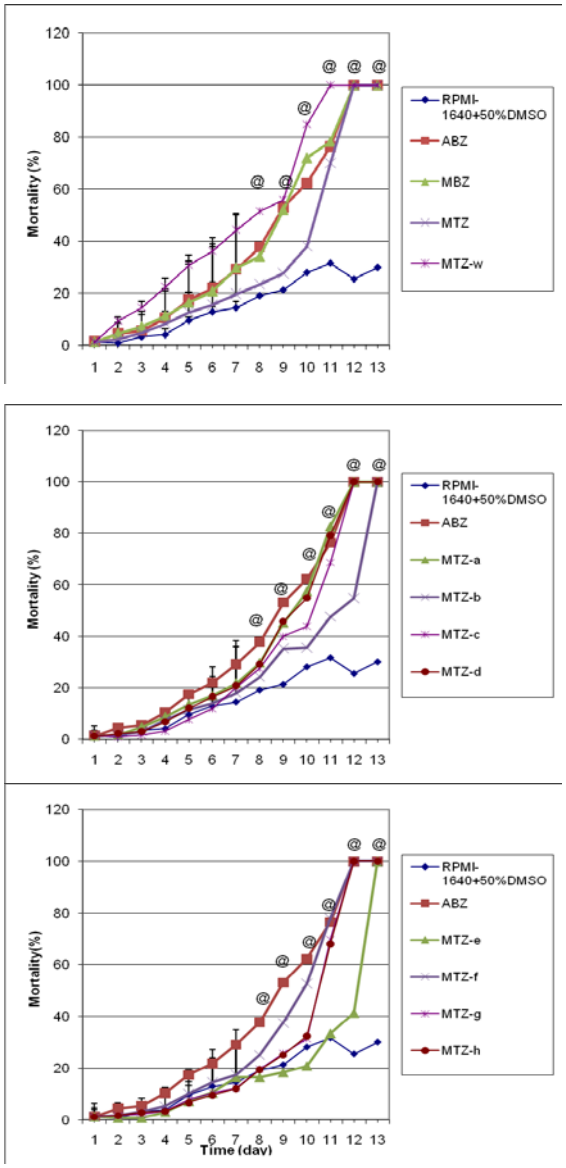

Figure 3. Mean percent mortality rates of freshly cultured *E. granulosus* PSCs with RPMI-1640 and treated with 12.5 µg/ml ABZ, MBZ, MTZ or its derivatives. " @: standard deviation was not placed because the values represent only one or two observations".

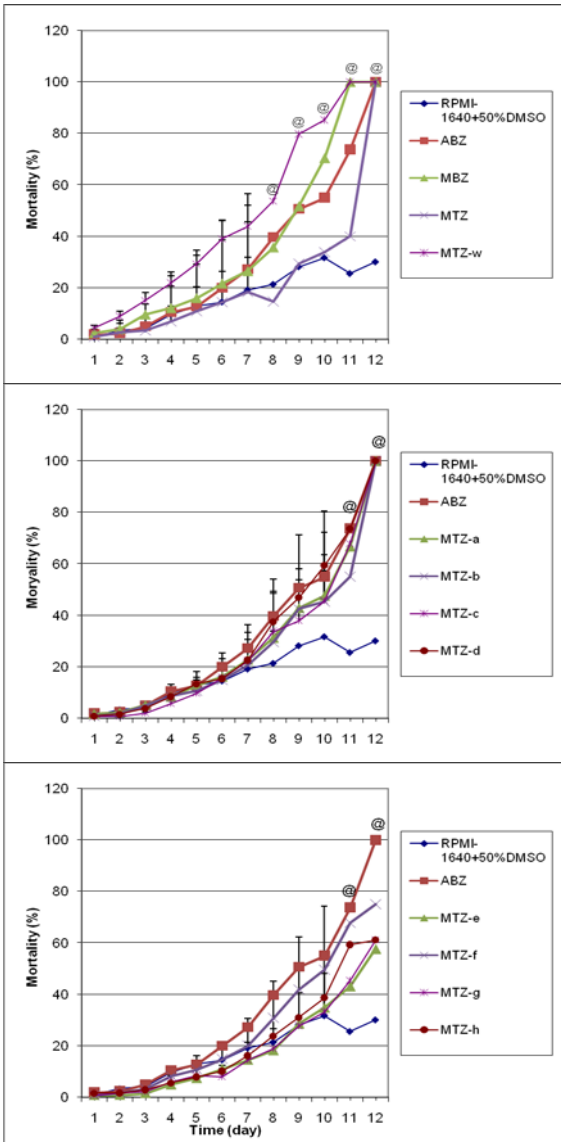

Figure 4. Mean percent mortality rates of freshly cultured *E. granulosus* PSCs with RPMI-1640 and treated with 6.25 µg/ml ABZ, MBZ, MTZ or its derivatives. " @: standard deviation was not placed because the values represent only one or two observations".

Moreover, total mortality always occurred one day earlier upon the use of MTZ-w compared to that when PSCs were exposed to ABZ. Mortality of cultured PSC in the presence of ABZ increased slowly during the first 8 days, while in the presence of MTZ-w it increased steadily in form of straight line during the same period. Thus, the death of 50% of cultured PSCs due to MTZ-w occurred at least one day prior to their exposures to comparable concentrations of ABZ or MBZ, respectively (Figures 2-4). During the early periods, incubation with 25 µg/ ml MTZ-w resulted in 3-5 fold mortality rates that caused by the standard positive control drug ABZ as depicted in Figure 5.

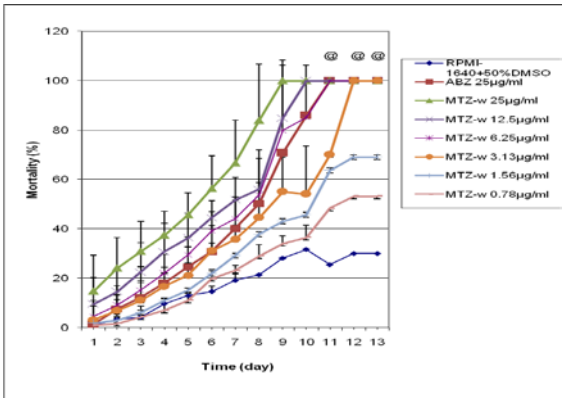

Figure 5. Mean percent mortality rates of freshly cultured *E. granulosus* PSCs with RPMI-1640 and treated with different concentration of MTZ-w compared with ABZ (+ control). "@: standard deviation was not placed because the values represent only one or two observations ".

For the three drug concentrations used, MTZ showed poor protoscolicidal effect that was significantly less than that caused by MTZ-w or ABZ. However, incubation of cultured PSCs with MTZ-a, MTZ-b and MTZ-d caused significantly less mortality values than those caused by MTZ-w but were closer to those caused by the standard positive control drug ABZ (Figures 2-4). In contrast, incubation with the three different concentrations of MTZ-c and MTZ-f was less effective in killing PSCs and MTZ-e, MTZ-g and MTZ-h were the least effective compared to other MTZ derivatives and standard drugs used.

Figure 5 shows that the protoscolicidal effect of MTZ-w followed a concentration gradient and the most effective was at concentration of 25 µg/ml and the lowest at a concentration of 0.78 µg/ml. Evidently, incubation with an MTZ-w concentration as low as 6.25 µg/ml was more effective in killing PSCs than that caused by ABZ at a concentration of 25µg/ml.

Light microscopy of *in vitro* cultured stages incubated with various drugs and MTZ-derivatives for 14 days reflected the relative detrimental changes caused by these compounds. The greatest morphological changes which included disruption of scolex hooks, deformation of suckers, and disintegration of the tegument was seen in case of PSCs exposed to MTZ-w or ABZ (Figure 6). Less drastic changes in form of dentated suckers, disrupted hooks and tegument were observed when MTZ-a, MTZ-b and MTZ-d were used. The use of MTZ-c caused tegumental and scolex changes which were intermediate between those caused by the above mentioned compounds in one hand and those caused by MTZ-e, MTZ-g and MTZ-h which were the least effective (Figure 6). Incubation of cultured PSCs to MTZ-a, MTZ-f and ABZ, appeared to shift PSCs differentiation into a globose shape. The degree of degenerative changes that included disruption of hooks, rupture of tegument and peri-tegumental accumulation of disrupted tissue increased with time following incubation with various compounds.

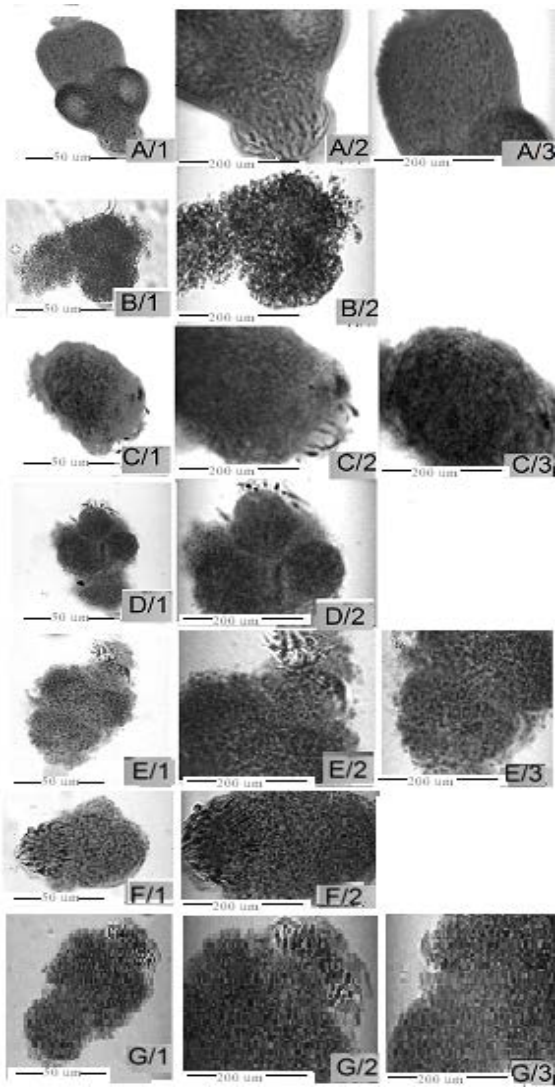

Figure 6. Light microscopy of 14 day old cultures of evaginated PSCs in RPMI-1640 incubated with 25µg/ml ABZ,MTZ or its derivatives in 25µg/ml: (A) RPMI-1640 represent negative control (A/1), (A/2) normal suckers and hooks, (A/3) body tegument; (B) ABZ: (B/1) ,(B/2) dentated suckers, disrupted hooks and disrupt body tegument; (C) MTZ (C/1) , (C/2) dentated suckers and disrupted hooks, (C/3) disrupted body tegument; (D) MTZ-w (D/1) , (D/2) dentated suckers, disrupted hooks and disrupted body tegument; (E) MTZ-a, MTZ-b or MTZ-d: (E/1) , (E/2) dentated suckers and disrupted hooks, (E/3) disrupted body tegument; (F) MTZ-c or MTZ-f: (F/1) , (F/2) dentated suckers and disrupted hooks, (F/3) disrupted body tegument; (G) MTZ-e, MTZ-g or MTZ-h: (G/1), (G/2) dentated suckers and normal hooks (G/3) disrupted body tegument.

3.2. Effect of MTZ and its derivatives on 30 day old cultured stages in vitro

The effects of ABZ and MTZ drugs as well as MTZ derivatives (all at 25 µg/ml concentration) on 30 day old cultured stage(S5 developmental stage) were followed for an additional 14 days. The metacestode stages in cultures treated with ABZ, MTZ-w, MTZ-a, MTZ-b, MTZ-d and MTZ-f revealed dentated suckers and disruption of both hooks and tegument. Less detrimental effects were observed using MTZ and other derivatives.

3.3. In vitro effects of MTZ and its derivatives on secondary developing HC in mice

Figure 7 displays typical ultrastructural effects of ABZ drug and MTZ-w on metacestodes cysts that were incubated in RPMI-1640 containing 25 µg/ml of each compound. During culturing and incubation with these compounds, HC regressed in size. Under SEM, the wall of HC incubated with RPMI-1640 appeared intact with smoothly lined LL and GL with intact tegument. In contrast, ABZ treated HC showed damaged GL, fragmentation of underlining tissue and scaling of LL with oval depressions that appear to lead to the involution and regression in HC size. HC incubated in MTZ-w revealed greater dentated damage in GL and more patchy LL with many deep depressions than those seen in cysts treated with ABZ.

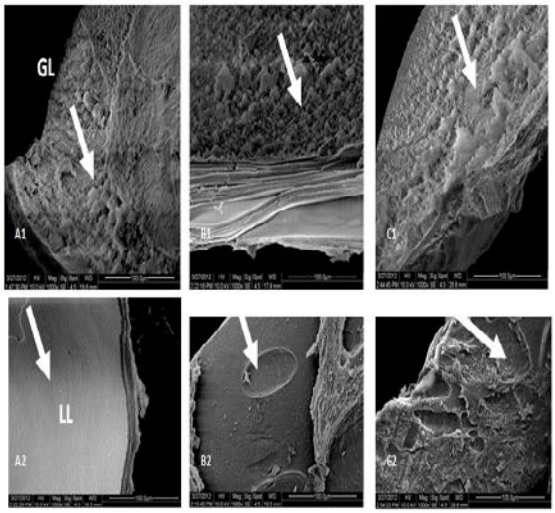

Figure 7. Scanning electron microscopy of laminated (LL) and germinal layer (GL) of secondary hydatid cysts developing in BALB /C mice 14 weeks post subcutaneous inoculation with PSCs: Cysts were incubated for 21 days in following media and compounds: RPMI-1640 as negative control showing intact GL (A1) and LL (A2) with remnants of host tissues towards the periphery of LL; RPMI- 1640 containing 25 µg/ml ABZ as positive control showing scaling of LL (B1), oval depressions and damaged GL with fragmented cellular elements (B2); RPMI- 1640 containing 25µg/ml MTZ-w showing carpet like appearance of GL with dentated endings (C1), and fragmentation of LL with many deep depressions and patchy appearance (C2).

4. Discussion

The present study documented for the first time the effects of several Schiff based and non-Schiff based MTZ derivatives on cultured *E. granulosus* PSCs and subsequent *in vitro* cultured stages. Indeed, one of the non-Schiff based MTZ derivative, MTZ-w, revealed remarkable activity and showed more protoscolicidal activity than ABZ, the drug of choice in CE treatment, even at one fourth the concentration of the latter drug. The mortality of PSCs and metacestode stages exposed to MTZ-w was about twice than ABZ at the same concentration and exceeded three times that of ABZ during early periods of exposure. Moreover, the damaging effects on hydatid cyst LL and GL incubated with MTZ-w was more than that on those incubated with ABZ at the

same concentration. In terms of molarity, exposure of PSCs and other *in vitro* cultured metacestodes to 25µg/ml concentration of MTZ-w or ABZ is equivalent to 110 and 94 µM solutions, respectively. Taking molarity into consideration does not change the comparative parasiticidal effects of these two compounds *in vitro*. MTZ-w remains significantly more effective than ABZ. Even the exposure of cultured metacestodes to as low as 27.5 µM solution of MTZ-w was significantly more lethal than that caused by exposure to 90 µM solution of ABZ.

Some other Schiff based MTZ derivatives, particularly MTZ-a, MTZ-b and MTZ-d showed protoscolicidal effects and mortality values close to those caused by the standard positive control drug, ABZ. These, in addition to the most potent MTZ-w, are thus important candidates for assessment as alternatives for ABZ both *in vitro* and *in vivo*. In contrast, MTZ itself does not seem to be a suitable drug against CE as it showed a much less protoscolicidal than ABZ.ABZ and MTZ must have different modes of action from that of MTZ-w which showed significant activity against PSCs and other cultured stages. MTZ-w, is an imidazole benzeyldehyde analogue, having imidazole ring as in ABZ [Methyl 5-propylthio-2-benzimidazole carbamate]. However, MTZ-w (4-[2-(2-methyl-5-nitro-1H-imidazol-1-yl) ethyoxy] benzeyldehyde) has 2-methyl-5-nitro with *para*-aldehyde benzene ring. Whether there is a synergistic effect of MTZ-w nucleus and the benzyldehyde group that renders it more effective on cultured PSCs than ABZ remains to be investigated. The activity of ABZ includes disruption of glucose uptake by inhibition of ß-tubulin of the endoplasmic reticulum and mitochondria of parasite GL (Polat *et al*., 2009). As with MTZ (Halloran *et al*., 2010; Lofmark, *et al*., 2010), the activities of Schiff bases is comparable to MTZ which suggests a similar mechanism of action. The differences in protoscolicidal activity between the several MTZ derivatives may reflect differences in stability and transport properties.

The *in vivo* dose of ABZ for chemotherapy against CE in human and livestock is 50 mg/kg body weight. Lower dosages of ABZ were given after the surgical treatment for maintenance purposes (Moreno *et al*., 2001; Adas *et al*., 2009; Creul *et al*., 2012). In the present study, the doses that were chosen to test the effects of ABZ and the various other drugs and MTZ derivatives in *in vitro* cultures of PSCs and subsequent stages were 25 µg/ml or lower. It should be pointed that lower dosages that prove effective against CE are more beneficial than higher dosages. In addition to decrease in cost, fewer side effects are expected with the use of lower dosages. Although the *in vivo* effect of MTZ derivatives, particularly MTZ-w requires further intensive assessment, comparisons of their effects on *in vitro* cultured metacestode stages with those caused by standard drugs are important initial steps towards searching for effective and safe drug alternatives. Thus, MTZ-w and other derivatives that showed sufficient *in vitro* parasiticidal activity at lower dosages should be followed further for potential use as chemotherapeutic drugs. Moreover, the fast action showed by MTZ-w and some other derivatives is of great importance. Fast action lowers the number and volume of drugs for treatment (Taylor *et al*., 1990; Todorov *et al*., 1992).

Cultures of PSCs reaching 30 days old stage are useful to assess the effect of the compounds on developing parasite stages. The effect of MTZ derivatives on this stage was studied after 14-days of incubation with single dose of 25µg/ml and MTZ-w showed the greatest detrimental effect. However, daily follow up is needed to compare the effect of these compounds and the timing needed to reach total (100%) parasiticidal effect. This should be carried out on various pre-segmentation and post- segmentation stages as well as adult worms. If proved effective, drug development against the developing and adult parasite stages in the dog definitive host are valuable. It should be pointed out that the experimental set up of *in vitro* culturing of PSCs and metacestode stages was done in a microenvironment where oxygen was in excess. MTZ and possibly its derivatives normally function under anaerobic or low oxygen tension conditions. As *Echinococcus granulosus* metacestode stages possess both aerobic and anaerobic respiratory systems (Cue *et al*., 2013), there is urgent need to explore further the effect of MTZ derivatives, particularly MTZ-w on *in vitro* cultured stages that are maintained under low oxygen microenvironment.

The target of MTZ-w and other effective derivatives on PSCs appear to be the tegument with subsequent effects on suckers and hooks. Tegument disruption, sucker collapse, and hook rupture were all noted using the most effective MTZ-w compound in addition to ABZ. The loss of rigidity and the size reduction of treated HCs with ABZ or MTZ-w may be due to changes in osmolarity inside and outside HC layers as a result of drug internalization through the cyst wall. Scanning electron microscopy results showed additional evidence about disruptive action of MTZ-w on HC. Here, we provide strong evidence of its potential as an anti-helminthic compound using the *E. granulosus* model. These findings build on the uniquely wide spectrum of this compound as antiprotozoal and antimicrobial activity (Gavidia *et al*., 2009; Abu Shaireh *et al*., 2009; Saadeh *et al*., 2010). There is an urgent need to examine the chemotherapeutic potential of this compound i*n vivo* using the mouse secondary hydatidosis model. This is a prerequisite for further studies on its toxicity, side effects, and bioavailability.

Acknowledgements

We thank Dr. Maen Abdel-Hafez and Dar Al-Hikmah Pharmaceutical Company, Amman, Jordan for providing albandazole and mebendazole compounds used in this study. Thanks to Mr. Tareq Ramadneh for technical help. This work was supported by a grant from the Research Council, Deanship of Scientific Research and Graduate Studies, Yarmouk University, Irbid, Jordan.

References

Abu Shaireh E, Saadeh H, Mosleh I, Al-Arif M and Mubarak M. 2009. Metronidazole derivatives as antiparasitic agents. *European Patent Applications*; **EP 2 085 394 A2**.

Adas G, Karatepe O, Altiok M, Battal M, Bender O, Ozcan D, *et al*. 2009. Diagnostic problems with parasitic and non-parasitic splenic cysts. *BMC Surg*., **9**:1186-1192.

Ceballos L, Elissondo M, Bruni S, Denegri G, Alvarez L, and Lanusse C. 2009. Flubendazole in cystic echinococcosis therapy:pharmaco-parasitological evaluation in mice. *Parasitol Int*., **58**:354-358.

Creul C, Codreanu1 R, Mastalier B, Popa1 I, Cordo M, Beuran D, *et al*. 2012. Albendazole associated to surgery or minimally invasive procedures for hydatid disease– how much and how long? *Chirurgia*, **107**:15-21.

Cui SJ, Xu LL, Zhang T, Xu M, Yao J, Fang CY*et al*. 2013. Proteomic characterization of larval and adult developmental stages in *Echinococcus granulosus* reveals novel insight into host-parasite interactions. *J Proteomics*, **84**:158-75.

Da Silva A. 2010. Human echinococcosis: a neglected disease. *Gastroenterol Res Pract*., Article ID 583297.

Eckert J and Deplazes P. 2004. Biological, epidemiological, and clinical aspects of echinococcosis, a zoonosis of increasing concern. *Clin Microbiol Rev*., **17**:107–135.

Gavidia C, Gonzalez A, Lopera L, Jayashi C, Angelats R, Barron E, Ninaquispe B, Villarreal L, Garcia L, Verastegui M and Gilman R. 2009. Evaluation of nitazoxanide and oxfendazole efficacy against cystic echinococcosis in naturally infected sheep. *Am J Trop Med Hyg*., **80**:367–372.

Gold, D. 1997. Assessment of the viability of *Schistosoma mansoni* schistosomula by comparative uptake of various vital dyes. *Parasitol Res.*, **83:** 163-169.

Halloran E, Hogan A and Mealy K. 2010. Metronidazole-induced pancreatitis. *HPB Surg.*, Article ID 523468.

Hemphill A and Muller J. 2009. Alveolar and cystic echinococcosis: towards novel chemotherapeutical treatment options. *J Helminthol*., **83**: 99–111.

Hijjawi N, Abdel-Hafez S and Al-Yaman F. 1992. *In vitro* culture of the strobilar stage of *E. granulosus*of sheep and donkey origin from Jordan. *Parasitol Res.*, **78**: 607-616.

Liu, CS, Zhang, HB, Yen, JH, Jiang, B and Han XM. 2013. *Echinococcus granulosus*: suitable *in vitro* protoscolices culture density. *Biomed Environ Sci*. **26:** 912-915.

Kakru D, Sofi B and Assadullah S. 2008. Novel route of injection in experimental model of hydatid disease. *Indian J PatholMicrobiol*., **51**: 373-375.

Kern P. 2006. Medical treatment of echinococcosis under guidance of good clinical practice (GCP/ ICH). *Parasitol Int*., **55** (Suppl 2): 273-282.

Lofmark S, Edlund C and Nord C. 2010. Metronidazole is still the drug of choice for treatment of anaerobic infections. *Clin Infec Dis*., **50**:16–23.

McManus, D. 2010. Echinococcosis with particular reference to Southeast Asia. *Adv Parasitol*., **72**:267-303.

Meyer M and Olsen O. 1980. **Essentials of Parasitology**. Wm C Brown Company, Dubuque, Iowa, p. 236.

Moreno M, Paris M, Casado N and Caabiaro F. 2001. Praziquentel and albendazole in the combined treatment of experimental hydatid disease. *Parasitol Res.*, **87**: 235-238.

Moro P and Schantz P. 2009. Echinococcosis: a review. *Int J Infec Dis*., **13**:125-133.

Polat E, Aslan M, Cakan H, Saribas S, Ipek T and Kocazeybek B. 2009. The effects of albendazole and povidone iodine for antimicrobial agents and chemotherapy of hydatid cysts protoscoleces, *in-vitro* and *in–vivo*. *Afr J Microbiol Res*., **3**:743-746.

Saadeh H, Abu Shaireh E, Mosleh I, Al-Bakri A and Mubarak M. 2011. Synthesis, characterization and biological activity of Schiff bases derived from metronidazole. *Med Chem Res*., **21**: 2969-2974.

Saadeh H, Mosleh I, Al-Bakri A and Mubarak M. 2010. Synthesis, characterization and antimicrobial activity of new 1,2,4- triazole-3- thiol metronidazole derivatives. *Monatsh Chem*., **141**:471-478.

Smyth J. 1967. Studies on tapeworm physiology. XI. *In vitro* cultivation of .*Echinococcus granulosus* from protoscolex to the strobilar stage. *Parasitology*, **57**:111-133.

Taylor D, Morris D and Richards K. 1990. Perioperative prophylactic chemotherapy of *Echinococcus granulosus*: determination of minimum effective length of albendazole therapy *in vitro* protoscoleces culture. *HPB Surg*., **2**:159-164.

Todorov T, Mechkov G, Vutova K, Georgiev P, Lazarova 1, Tonchev Z, *et al*.1992. Chemotherapy of human cystic echinococcosis: comparative efficacy of mebendazole and albendazole.*Ann Trop Med Parasitol*., **86**:59-66.

Vuitton, D. 2009. Benzimidazoles for the treatment of cystic and alveolar echinococcosis: what is the consensus? *Expert Rev Anti Infect Ther*., **7**:145-149.

Wanner G, Schroeder-Reiter E and Formanek H. 2005. 3D analysis of chromosome architecture: advantages and limitations with SEM. *Cytogenet Genome Res*., **109**:70–78.

JJBS

Volume 7, Number 3, September .2014
ISSN 1995-6673
Pages 195 - 198

Jordan Journal of Biological Sciences

Measurements of Homogentisic Acid levels in Alkaptonuria Patients Using an Optimized and Validated Gas Chromatography Method/Mass Spectrometry

Sameeh A. Al-Sarayreh[1], Ibrahim N. Al-Tarawneh[2], Mohammed S. Al-Sbou[3,*], Eman M. Albatayneh[4], Jehad M. Al-Shuneigat[1] and Yousef M. Al-saraireh[2]

[1]*Department of Biochemistry and Molecular Biology, Faculty of Medicine, Mutah University;*

[2]*Department of Chemistry, Al-Balqa Applied University,*

[3]*Department of Pharmacology, Alkaptonuria Research Office, Faculty of Medicine, Mutah University,*

[4]*Department of Microbiology and Immunology, Faculty of Medicine, Mutah University, Jordan*

Received: April 1, 2014 *Revised: June 4, 2014* *Accepted: June 10, 2014*

Abstract

Alkaptonuria is a very rare genetic disorder, characterized by a lack of homogentisate dioxygenase and causes accumulation of homogentisic acid. Clinical manifestations include dark urine, dark-black pigmentation of connective tissues (ochronosis), and arthritis of large joints and spine. The disease is usually diagnosed in adulthood by assessment of signs and symptoms of ochronosis. Confirmation of suspected diagnosis can be achieved by quantitative measurements of homogentisic acid levels. In this study, homogentisic acid was analyzed in 17 alkaptonuria patients. After liquid-liquid extraction, the analyte was determined by Gas Chromatography/ Mass Spectrometry (GC-MS) method. For quantitation purposes, external calibration was applied first, regression coefficient of ≥ 0.995 indicated the linearity in the concentration range of 1-100 ng/µl. The instrumental detection limit (IDL) and lower limit of quantitation were 3.82 and 12.7 µg/L, respectively. Recovery rate was ≥ 89%. Precision given as relative standard deviation (RSD) ranged from 3 – 10 %. The results showed that the concentration of homogentisic acid ranged from 0.46 to 1.5 g/24 hours.

Keywords: Homogentisic acid, Black urine, Ochronosis, GC-MS, Jordan.

1. Introduction

Alkaptonuria (AKU) was designated by Sir Garrod as the first inherited metabolic disease (Garrod, 1908). AKU is a rare autosomal recessive disease caused by a deficiency of a specific enzyme, homogentisate 1,2 dioxygenase (HGD), leading to accumulation of homogentisic acid (HGA) (La Du, 1958). Most of the HGA is excreted in urine, and some is deposited in connective tissues as a melanine-like polymer in a process known as ochronosis (Zannoni *et al.*, 1969). The pathophysiological mechanism of AKU is still unclear. Oxidative stress and amyloid formation may play a fundamental role in AKU. Indeed, recent studies have shown the presence of serum amyloid A (SAA) and serum amyloid P (SAP) in vitro and ex vivo AKU models and highlighted AKU as a secondary amyloidosis (Millucci *et al.*, 2012; Braconi *et al.*, 2013). The clinical features of AKU are characterized by homogentisic aciduria, bluish-black discoloration of connective tissues and arthropathy of weight-bearing joints such as hips and knees and spondyloarthropathy (O'Brien *et al.*, 1963). Complications of the disease include stones formation in kidneys, prostate, gall bladder and salivary glands, rupture of tendons, ligaments, muscles and cardiovascular manifestations (cardiac arrhythmias, aortic valve disease) (Phornphutkul *et al.*, 2002). AKU severity score index (AKUSSI) have been developed for the first time as an assessment tool to quantitate disease severity, to compare the severity between AKU patients and to measure the progression of the disease (Ranganath, 2011; Cox, 2011).

AKU can be diagnosed at birth. The earliest clinical signs are dark urine and discoloration of nappies. However, some AKU patients are asymptomatic, and the majority of patients are diagnosed late in the third decade of life when they are affected by the ochronotic arthropathy (Ranganath *et al.*, 2013). The urine of the

* Corresponding author. e-mail: malsbou@mutah.edu.jo.

AKU patient turns dark-black when exposed to air. This reaction can be accelerated by alkalinizing urine (Castagna *et al.*, 2006). Other screening tests include darkening of urine after adding ferric chloride (FeCl3). Confirmation of diagnosis is established by the identification and quantification of urinary HGA using chromatographic techniques, such as gas chromatography mass spectrometry (GC-MS) and high-performance liquid chromatography (HPLC) (Abdulrazzaq *et al.*, 2009; Bory *et al.*, 1989).

The incidence of AKU around the world is as low as 1 in 250,000; however, high incidences have been reported in Slovakia and the Dominican Republic (1 in 19,000) (Srsen *et al.*, 1978; Milch, 1960). The incidence of AKU in Jordan is unknown; however, recent studies have identified 60 cases with AKU. Most of these cases were identified in a small village in south Jordan, were nine AKU patients were diagnosed in the same family. The high rate of this rare genetic disease in Jordan is believed to be due to high rates of consanguineous marriages (Al-sbou *et al.*, 2012a; Al-sbou *et al.*, 2012b). Several methods have been used to measure HGA in subjects with AKU include chromatographic techniques such as spectrophotometric methods, high-performance liquid chromatography (HPLC), and gas chromatography mass spectrometry (GC-MS) (Seegmiller *et al.*, 1961; Borry *et al.*, 1989 Markus *et al.*, 2001). The aim of this study is to describe the determination of urinary HGA using a sensitive and specific GC-MS method.

2. Materials and Methods

2.1. Chemicals and Standard Solutions

m-Methoxy-acetophenone (internal standard-I.S.) and N, O-bis(trimethylsilyl) trifluoroacetamide (BSTFA) used for derivatization were purchased from Across Chemical (USA). Homogentisic acid was supplied from Sigma Aldrich/ Germany. The following solvents of GC-grade were purchased from Riedel-de Haën (Germany): n-Hexane, ethyl acetate, methanol and pyridine.

2.2. Sampling and Sample Preparation

Twenty four hours urine samples were collected from 17 AKU patients. Those patients were registered in the Jordanian Society of Alkaptonuria and were diagnosed having the disease based on results of laboratory investigations and clinical assessment. First, urine samples were left standing at room temperature for 48 h and were observed for changing the color. Urine samples of AKU patients turned dark-black upon standing as shown in Figure 1. Second, ferric chloride test was performed by adding one drop of ferric chloride solution and was positive if a transient blue color was observed. Clinical assessment and radiological examinations of patients were conducted to confirm the presence of signs and symptoms of ochronosis.

N,O-bis (trimethylsilyl) trifluoroacetamide (BSTFA) is mostly used as derivatizing agent and it has a high reactivity. The derivatization of homogentisic acid compound for GC-MS analysis was performed according to (Zafra *et al.*, 2006) with slight modification, in which a mixture of 20: 5:25 (v/v/v) BSTFA-pyridine-ethyl acetate (containing homogentisic acid) is allowed to stand for 2 min at room temperature. The procedure is enough to get adequate derivatization. 1.0 mL of liquid urine sample was transferred into polypropylene test tube and 1g NaCl, 200µL of 5M HCl and 6mL ethyl acetate were added to the sample. The last mixture was shaken well for 10 min and centrifuged at 4000 rpm for 3min, and then the upper layer (ethyl acetate) transferred into vacuum test tube. The extracts were evaporated to dryness using a gentle stream of nitrogen. The residues were reconstituted in750 µL of 6.65 mg/L internal standard dissolved in ethyl acetate using. 250µL of the previous solution were mixed with50µL pyridine and 200µL BSTFA. The mixture was shaken well for two minutes in order to derivatize the phenolic compounds. Finally, a volume of 1µL of the prepared solution was injected into GC-MS instrument.

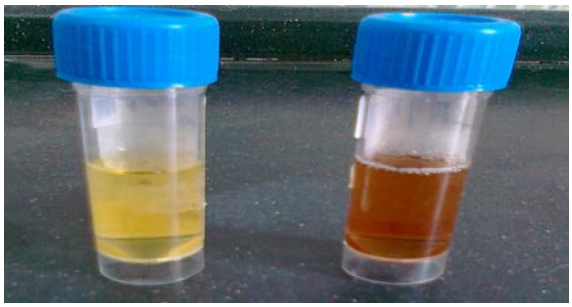

Figure 1. Urine samples from AKU patient, fresh (left) and after 48 hours (right).

2.3. GC-MS Analysis

The gas chromatographic analysis was performed using an Agilent 6890 Series II. A gas chromatograph fitted with an auto sampler injector. A capillary column HP-5 fused silica column (30m × 0.25mm, film thickness 0.25µm,(5%)-biphenyl-(95%)-dimethylsiloxane copolymer) was used. A silanized injector liner split/splitless (2mm I.D.) was used. Detection was carried out with a 7683 mass-selective single quadrupole detector (Agilent Technologies). The injector temperature was 250°C. The oven temperature was held at 80°C for 3 min, and then increased to 240°C at a heating rate of 13°C min^{-1}, and the temperature was held for 20 min. The detector temperature was 280°C. The carrier gas used was helium (purity 99.999%) at a flow rate of 1.0mL min^{-1}. The samples were injected in the splitless mode and the splitter was opened after 7 min (delay time). The sample volume in the direct injection mode was 1µL. The ion energy used for the electron impact ionization (EI) mode was 70eV. The mass range was scanned from 150–550m/z. Single ion monitoring (SIM) acquisition mode was used (Deeb *et al.*, 2012). The mass spectrum showed the molecular ions at m/z 384, 341 at 14.2 min and 150, 135 at 9.5 min which corresponds to the correct molecular formula C17H32O4Si3 (homogenistic acid) and C9H9O2 (I.S.), respectively as shown in Figure 2.

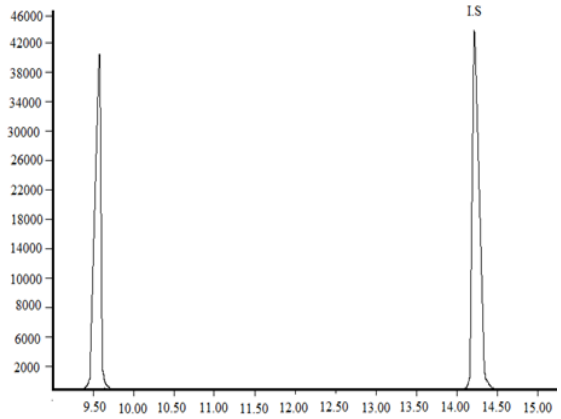

Figure 2. GC-MS chromatogram of the 5.0 mg/L level homogenisctic acid (9.5 min) with 6.65mg/L IS (14.2min)

2.4. Analytical Quality Assurance

Standard mixture solutions of 1.0, 5.0, 20, 50, 100 mg/L of the derivatized homogentisic acid with 6.65mg/L IS were prepared in order to define the linear working range. Internal standard method was preferred in order to correct any loss of phenolic compound (homogentisic acid) during sample preparation. Regression coefficient (r2) was in all cases > 0.995, indicating the linearity of the calibration function in this concentration ranges.

Extraction recovery of homogentisic acid was determined for urine samples at 3 spiking levels, 1, 20, 100µg/L, where the average recovery was found between 89 – 105%. Additionally, blanks were analyzed for background concentration. Precision of the method for homogentisic acid, calculated as relative standard deviation (RSD) was ranged between 2-9 %. The instrumental detection (IDL) was estimated form injections of standard solutions successively diluted until reaching a concentration level corresponding to a signal-to-noise ratio of 3. The method quantification limits (MQL) was determined form spiking urine sample, as the minimum detectable analyte concentration, which give signal-to-noise ratio of 10.

3. Results and Discussion

AKU patients in this study were 12 males and 5 females with a mean age of 29 years (age range 5-53 years). All patients had a history of dark urine since birth. Ferric Chloride test was positive for all patients. Quantitative measurements of urinary HGA showed that all patients excreted large quantities of HGA, the mean was (0.95 g/24 h). A GC/MS representative chromatogram for a real sample of AKU patient is shown in Figure 3.

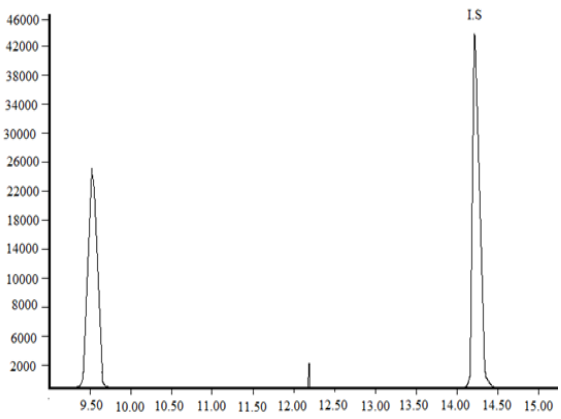

Figure 3. Representative GC-MS chromatogram for a real sample of AKU patient

There was a marked variation in the concentration of HGA excreted between different AKU patients (a 3-fold variation) and the range was (0.46 - 1.5 g/24 h) (Table 1). This variation could be due to different dietary protein content between different individuals.

Table 1. Urinary HGA levels in 17 alkaptonuria patients

Case No	Age (Years)	Sex	Urinary HGA g/24 hrs
1	5	M	0.46
2	5	M	0.53
3	6	M	1.17
4	8	F	0.8
5	13	M	1.2
6	24	F	1.37
7	26	M	0.51
8	33	M	0.9
9	34	M	0.8
10	37	M	1.47
11	37	M	0.79
12	39	M	1.1
13	44	M	0.7
14	44	F	0.77
15	44	F	1.5
16	44	M	0.88
17	53	F	1.1

M=male, F=female

AKU is a very rare genetic condition and in most cases it is misdiagnosed at childhood. Few signs and symptoms of this disease appear before the 3rd decade of life, apart from passage of dark urine.

The reason for the delay appearance of ochronotic manifestations is unknown. Therefore, the majority of cases with AKU are recognized in adulthood (Ranganath *et al.*, 2013). Homogentisic acid is a normal intermediate in the metabolism of tyrosine and normal individuals do not excrete HGA because it is converted into maleylacetoacetic acid by the homogentisate 1,2 dioxygenase. AKU patients excrete high concentrations of HGA in urine (range 4-8 g/day) (Castagna *et al.*, 2006). Therefore, testing urine samples for the presence of HGA is crucial for establishing the diagnosis of AKU. Some screening tests can provide help in diagnosis of AKU such as darkening of urine after addition of sodium hydroxide, and ferric chloride test. This test can be used to detect metabolites in urine samples of patients with inborn error of metabolism diseases such as AKU (Frohlich *et al.*, 1973). The basis of this test is that phenols of HGA form a violet complex with Fe (III), which is intensely colored, therefore, the urine containing HGA yields a transient blue color after adding few drops of ferric chloride solution. In this study, this test was positive for all tested AKU patients, thus it can be used to help in screening for AKU. However, confirmation of diagnosis of AKU can be achieved by specific identification and quantification of HGA in urine and blood samples.

The present Gas Chromatography/ Mass Spectrometry method was developed for the determination of homogentisic acid in AKU patient's urine; in addition the derivatization of homogentisic acid compound was performed with a slight modification to Zafra's method (Zafra *et al.*, 2006). The main advantage of this method includes its high sensitivity, with MQL for HGA in urine as low as 12.7 µg/ L, also this method showed a very good separation of analyte (homogentisic acid) in less than 15 min.

4. Conclusion

The described method represents a useful and suitable analytical tool that can be used for diagnosing AKU and for the monitoring treatment effect on HGA levels in AKU patients.

References

Abdulrazzaq YM, Ibrahim A, Al-Khayat AI, Nagelkerke N and Ali BR. 2009. R58fs mutation in the HGD gene in a family with alkaptonuria in the UAE. *Ann Hum Genet*, **73**:125-30.

Al-sbou M, Mwafi N and Abu Lubad M. 2012b. Identification of forty cases with alkaptonuria in one village in Jordan. *Rheumat Int*, **32**:3737-3740**.**

Al-sbou M and Mwafi N. 2012a. Nine cases of Alkaptonuria in one family in southern Jordan. *Rheumat Int*, **32**:621–625.

Bory C, Boulieu R, Chantin C and Mathieu M.1989. Homogentisic acid determined in biological fluids by HPLC. *Clin Chem.*, **35**, 321-322.

Braconi D, Millucci L, Ghezzi L and Santucci A. 2013. Redox proteomics gives insights into the role of oxidative stress in alkaptonuria. *Expert Rev Proteomics*, **10 (6)**:521-535.

Castagna A, Giombini A, Vinanti G, Massazza G and Pigozzi F. 2006. Arthroscopic treatment of shoulder ochronotic arthropathy: a case report and review of literature. *Knee Surg Sports Traumatol Arthrosc.*,**14**:176-181.

Cox TF and Ranganath LR. 2011. A quantitative assessment of alkaptonuria. *J Inherit Metab Dis.*, **34**: 1153-1162.

Deeb AA, Fayyad MK and Alawi MA. 2012. Separation of polyphenols from Jordanian olive oil mill wastewater. *Chromatog Res Int.*, 1-9.

Frohlich J, Price GE and Campbell DJ. 1973. Problems in the laboratory diagnosis of alkaptonuria. *Clin Chem.*, **19(7)**: 770-773**.**

Garrod AE. 1908. The Croonian lectures on inborn errors of metabolism. Lecture II. Alkaptonuria. *Lancet*, **2**:73-79.

La Du BN, Zannoni VG, Laster L and Seegmiller JE. 1958. The nature of the defect in tyrosine metabolism in alcaptonuria. *J Biol Chem.*, **230**:251-260.

Markus AP, Swinkels DW, Jakobs BS, Wevers RA, Trijbels JM and Willems HL. 2001. New technique for diagnosis and monitoring of alcaptonuria: quantification of homogentisic acid in urine with mid-infrared spectrometry. *Analytic Chimica Acta*, **429**:287-292.

Milch RA. 1960. Studies of alcaptonuria: inheritance of 47 cases in eighty highly inter-related Dominican kinders. *Am J Hum Genet.*, **12**:76-85.

Millucci L, Spreafico A, Tinti L, Braconi D, Ghezzi L, Paccagnini E,Bernardini G, Amato L, Laschi M, Selvi E, Galeazzi M, Mannoni A, Benucci M, Lupetti P, Chellini F, Orlandini M and Santucci A. 2012. Alkaptonuria is a novel human secondary amyloidogenic disease. *Biochim Biophys Acta*, **1822 (11)**:1682-1691.

O'Brien WM, La Du BN and Bunim, JJ. 1963. Biochemical, pathologic, and clinical aspects of alkaptonuria, ochronosis, ochronotic arthropathy. *Am J Med*, **34**:813-823.

Phornphutkul C, Introne WJ, Perry MB, Bernardini I, Murphey MD and Fitzpatrick DL.2002. Natural history of alkaptonuria. *N Engl J Med.*, **347(26)**:2111-2121.

Ranganath LR and Cox TF. 2011. Natural history of alkaptonuria revisited: analyses based scoring systems. *J Inherit Metab Dis.*, **34 (6)**:1141-51.

Ranganath LR, Jonathan JC and Gallagher JA. 2013. Recent advances in management of alkaptonuria (invited review; best practice article). *J Clin Path.*, **66**:367-73.

Seegmiller JE, Zannoni VG, Laster L and La Du BN. 1961. An enzymatic spectropotometric method for the determination of homogentisic acid in plasma and urine. *J Biol Chem.*, **236**:774-777.

Srsen S, Cisarik F, Pasztor L and Harmecko L. 1978. Alkaptonuria in the Trencin District of Czechoslovakia. *Am J Med Genet.*, **2**:159-166.

Zafra A, Juárez MJB, Blanc R, Navalón A, González J and Vílchez JL. 2006. Determination of polyphenolic compounds in wastewater olive oil by gas chromatography-mass spectrometry. *Talanta***,70**:213–218.

Zannoni VG, Lomtevas N and Goldfinger S.1969. Oxidation of homogentisic acid to ochronotic pigment in connective tissue. *Biochimica Acta*, **177(1)**:94-105.

JJBS

Volume 7, Number 3, September .2014
ISSN 1995-6673
Pages 199- 203

Jordan Journal of Biological Sciences

Microscopic Analysis of *in vitro* Digested Milled Barley Grains: Influence of Particle Size Heterogeneity

Ghaid J.Al-Rabadi*

Department of Animal Production, Faculty of Agriculture, Mutah University, Al-Karak 61710 , Jordan

Received: May 21, 2014 Revised: June 12, 2014 Accepted: June 19, 2014

Abstract

In this study, Scanning Electron Microscopy (SEM) is used to characterize the structure of ground and whole barley grain before and after the exposure to *in vitro* enzymatic digestion at different incubation times (0, 0.5, 1, 2, 6 and 24 h). SEM analysis showed that digestion started to take place in barley grain fragments after 0.5 h of incubation time. SEM indicated that complete starch digestion is dependent on grain fragment size in barley. Starch digestion seems to be completed after 24 hours of digestion in small fragments of barley grains (<0.5 mm) which was not the case for larger fragment size (>1.0mm). In case of whole barley grain, SEM showed that alpha amylase was not capable of penetrating and diffusing through barley grain husk after 24 h of incubation. In conclusion, microscopic examination for *in vitro* digested milled and unprocessed barley fragments differ in particle size, indicating that the extent of starch digestion is dependent on fragment particle size.

Keywords: Scanning Electron Microscopy, Starch granules, *in vitro* digestion , Barley fragments

1. Introduction

Grains usually represent the main energy source in animal's diets where starch represents the main nutrient components (Svihus *et al.,* 2004). From a processing prospective, grains, such as barley, should be ground in order to facilitate further processing steps such as mixing and enhancing nutritive value by increasing digestibility (Al-Rabadi *et al.,* 2009). Hammer mill is widely used in the feed industry in order to mill grains as it is characterized by high production capacity and lower maintenance requirements (Amerah *et al.,* 2007). However, grains milled using hammer mill have been reported to produce wide variation in grain particle size (Audet, 1995). Heterogeneity of particle size within milled grains has been reported to influence nutrient digestibility even when the average particle size was the same (Wondra *et al.,* 1995). Within grain type, different grain fragment size, after being fractionated by sieving process, have been reported to posses different surface area per unit mass and different chemical composition (Al-Rabadi *et al.,* 2013). These factors have been reported to extensively influence the magnitude of starch digestion (Al-Rabadi *et al.,* 2012). Scanning electron microscopy (SEM) have been extensively used to track structural changes that occur into starch granules after being exposed to thermo mechanical treatments and amyloytic digestion (Srikaeo, 2008; Srikaeo *et al.,* 2006). The first objective of this study is to examine the influence of adding enzymes mixture (amylase, glucosidase, pepsin and proteases) in a sequence that mimic the digestion process *in vivo* with taking into consideration the heterogeneity of different size fragments of barley. A previous study reported that starch digestion of milled grains by alpha amylase is controlled by a diffusion process (Al-Rabadi *et al.,* 2009). However, this study aims at confirming the capability of alpha amylase to diffuse through barley grain husk using SEM.

2. Materials and Methods

2.1. Barley Grain Milling

Barely grains were milled using 4 mm hammer mill screen size when constant motor load was recorded. Ground and whole barley grains were collected and were sealed into plastic bags and stored at 4 ˚C until visual examination by using scanning electron microscopy and further being digested using in vitro starch digestibility method.

2.2. In vitro Starch Digestibility

In vitro starch digestion method was used as previously described by Al-Rabadi *et al.* (2009). In vitro digestion method was performed in a three-step enzymatic digestion to mimic digestion in the mouth, in the stomach and the small intestine in a closed system. Different digestion times (0, 0.5, 1, 2, 6 and 24 h) were used to simulate digestion process in monogastric animals and

* Corresponding author. : e-mail: ghaid78@yahoo.com.

young ruminates before weaning. The zero hour digestion was started at the start of the small intestinal simulation step (where most of starch digestion take place).

2.3. Scanning Electron Microscopy (SEM)

Milled barley grains fragments (before and after digestion at different incubation times) were placed onto aluminium stubs with carbon tabs. Fragments then were sputter coated (10-15 nm layer) of platinum using an Platinum Sputter Coater (model EIKO IB-5). Digested and undigested barley fragments were examined in either a JEOL 6300 or JEOL 6400 field emission scanning electron microscope. Micrographs were chosen by taking many pictures (i.e., 5 to 10 pictures) for the selected samples to obtain representative Scanning electron micrographs. The selected sample contains many barley grain fragments on the carbon tabs. Comparable appearance was selected as a representative picture. Many pictures (6-10 pictures) were taken at different magnifications to find any main structural difference at both grain fragment size level and starch granule size level.

3. Results and Discussion

Scanning electron micrographs for milled raw barley samples by using 4 mm hammer mill screen size is shown in Figure 1. Milling process resulted in breaking barley grains into different levels of fragment sizes that ranged from very fine particles to quarter and half broken grains (Figure 1). Previous studies showed that milling grains using hammer mill resulted in high heterogeneity in grain particle size distribution when compared with other milling equipments such as roller mill (Seerley *et al*., 1988; Douglas *et al.,* 1990; Audet, 1995). It can be also seen from Figure 1 that barley grains milled by using hammer mill produce spherical shape fragments. In his report, Kim (2002) reported that the hammer mill produces spherical shape grain fragments while the roller mill produces more rectangle grain fragments after milling.

The effect of alpha amylase on starch granules digestion, at different incubation times (0, 0.5, 1, 2, 6 and 24 h), using three enzymatic step models were examined (Figures 2, 3, 4, 5, 6, 7 and 8). As expected, there was no enzymatic activity on starch granules at 0 hour incubation time (Figure 2) although starch granules were exposed to salivary alpha amylase. It is hardly for any enzymatic digestion to take place on starch granules after 30 minutes of the incubation time (Figure 3). It has been previously shown that the enzymatic digestion by amylase is controlled by diffusion process through channels present on granules surface (Helbert *et al.,* 1996) and this may suggest that starch digestion may take place inside starch granules. Magnitude of diffusion coefficient for amylase has been previously quantified in barley starch granules (1.7 x 10-7 cm2 s-1) (Al-Rabadi *et al*., 2009). Extant of starch digestion for different grain fragment size, ranging from 0.045-2.8 mm, have been reported to range from 23-1%, respectively after a 30-minute incubation time (Al-Rabadi *et al.*, 2012).

Enzymatic digestion by amylase started to take place on starch granules surface after one hour incubation time (Figure 4). However, enzymatic activity by alpha amylase does not seem to be associated with every starch granules. On the other hand, digestion by alpha amylase seems to be associated with every starch granules after 2 h of incubation time (Figure 5). A number of holes on starch granules resulted from enzymatic activity increased as the incubation time progresses (i.e., digestion 6 h) as shown in Figure 6. Integrity of oval shape structure of starch granules starts to disappear after 24 h of incubation time for large fragment size (>1mm) as shown in Figure 7. However, starch granules in smaller fragment size disappeared after a 24-h digestion time, as shown in Figure 8. Complete starch digestion was achieved for barley fragment.

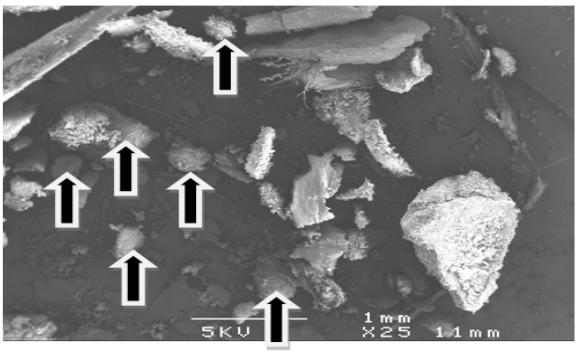

Figure 1. Raw milled barley grain fractions using 4 mm hammer mill screen size (heterogeneity of milled grain particle size range from very fine particles to half broken grains). Round oval shape particles indicated by black arrows.

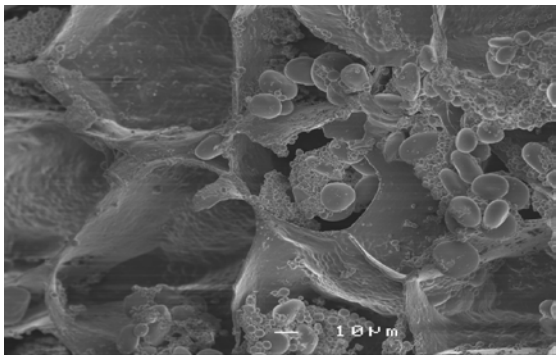

Figure 2. Undigested starch granules embedded in protein matrix.

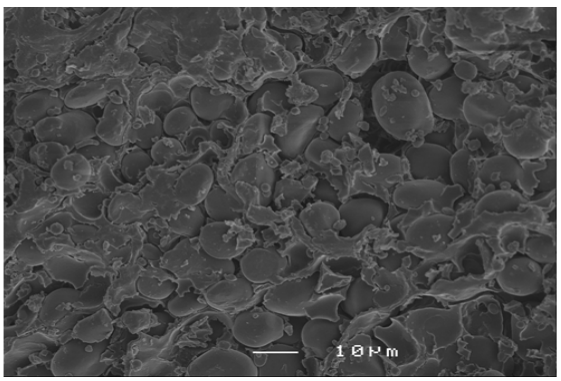

Figure 3. Digested starch granules after 0.5 h incubation time (no appearance for any enzymatic activity on starch granules). Image was taken from small fragment size (<0.5mm)).

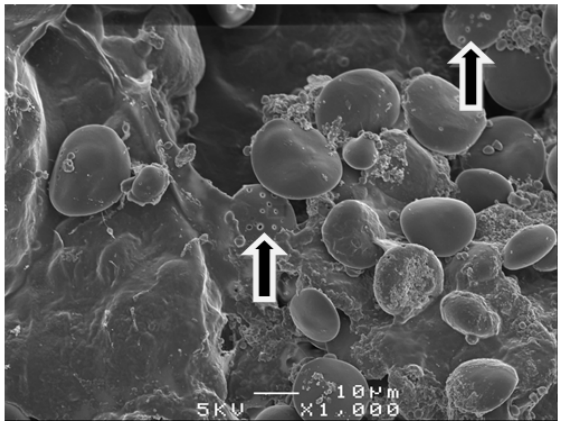

Figure 4. Digested starch granules after 1 h incubation time (initial enzymatic activity (holes) on certain starch granules as indicated by arrow). Image was taken from small fragment size (<0.5mm)).

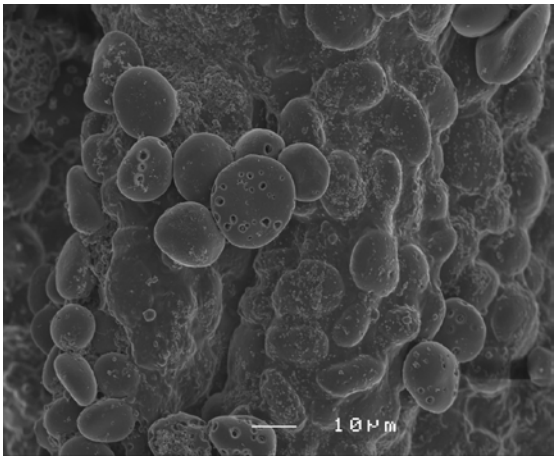

Figure 5. Digested starch granules after 2 h incubation time (initial enzymatic activity (holes) on most starch granules). Image was taken from small fragment size (<0.5mm))

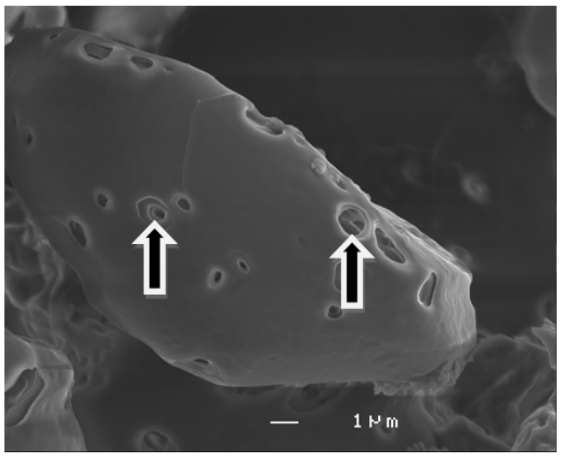

Figure 6. Digested starch granule after 6 h incubation time (increase the number and size of digestion holes compared to starch granules digested at 2 h incubation time as indicated by black arrows). Image was taken from small fragment size (< 0.5mm)).

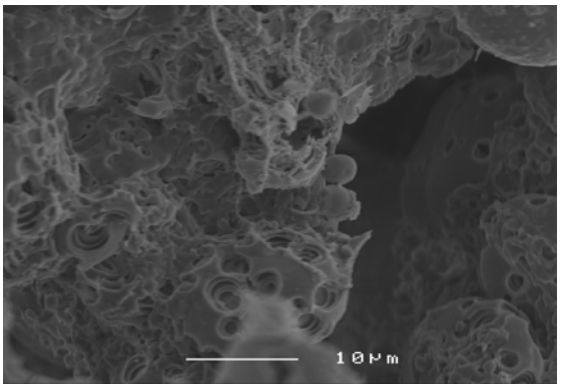

Figure 7. Digested starch granule after 24 h incubation time (increase the number and size of digestion holes). Image was taken from large fragment size (>1.0 mm)).

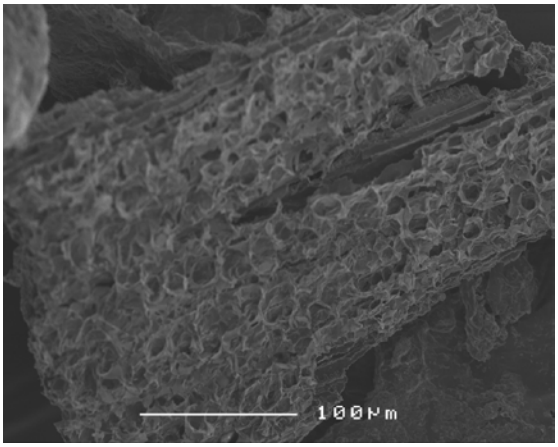

Figure 8. Absence of any starch granules after 24 h incubation time. Image was obtained from particles < 0.5mm.

It was found that the electron micrographic features of the granules after treatment with alpha amylase and glucosidase possesses synergistic influance (Matsubara *et al.,* 2004). The synergetic influences by both enzymes were explained by Sun and Henson (1990) and Robertson *et al.* (2006). The ability of the alpha-glucosidases to breakdown glucosidic bonds other than alpha-1,4- and alpha-1,6- that are present at the granule surface can eliminate bonds which were barriers to digestion by alpha-amylases. In addition, the presence of protease in the current in vitro digestibility method may enhance indirectly the synergy influence of both alpha amylase and glucosidase by increasing the exposure of starch granules to enzymatic digestion. It has been reported that the interaction between protein and starch granules can decrease the exposure of raw starch granules to enzymatic digestion by alpha amylase (Rooney and Pflugfelder, 1986). The interactions between the protein granules (size range 5–60 kDa) and starch may affect starch digestibility; it is important to take into consideration that protein digestion usually precedes starch digestion (Svihus *et al.,* 2005).

The size of the starch granules within grain type can influence the starch digestion process when examined using SEM. Large starches granules displayed massive degradation and were described by sever corrosion toward the radial axis of granule (Franco and Preto, 1992). On the other hand, small starch granules showed a surface

attrition and, later on, followed by solubilization (Franco and Preto, 1992). The difference in the behavior of starch digestion between small and large particles could be attributed to many factors. A previous study showed that large and small starch granules possess different chemical compositions and endothermic properties and thus possess a different enzymatic response to digestion by alpha amylase (Szczodrak and Pomeranz, 1991). Chiotelli and Le Meste (2002) reported that large starch granules have a lower water affinity due to more compact structure (i.e., higher crystallinity) than small starch granules and this could increase their susceptibility to enzymatic hydrolysis by alpha amylase. In addition, small starch granules

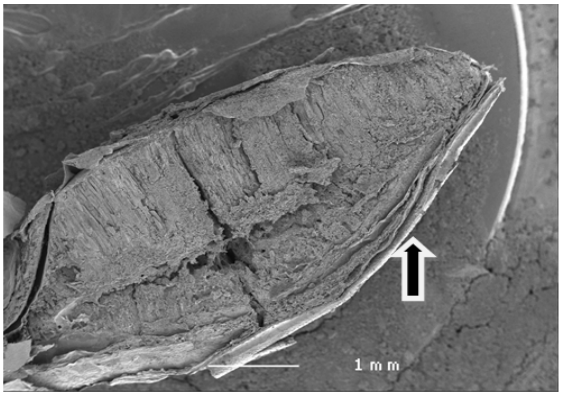

Figure 9. Cross section of whole barley grain after 24 h incubation time (barley grain husk is indicated by arrow).

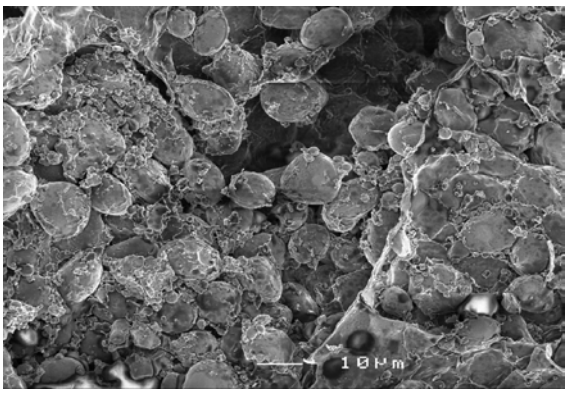

Figure 10. Absence of any enzymatic activity of whole barley grain .

have a higher surface area to weight ratio and this may suggest that alpha amylase binding to starch granules and the potential hydrolysis would be higher compared to large starch granules when all other factors being the same (Tester *et al.*, 2004).

In this study, the presence of large fragment size of barley fragments and the absence of fiber digesting enzymes may inhibit the synergetic influence of both alpha amylase and glycosidase. To confirm the capability of both alpha amylase and glycosidase to diffuse through barley grain husk which is mainly composed of cellulose, hemicelluloses and lignin (Adrados *et al*., 2005). Whole barley grain was incubated into digestion solution for 24 h to investigate whether any enzymatic digestion can take place (Figure 9). As shown in Figure 10, no enzymatic digestion took place on starch granules after cutting the whole barley grains into two halves, which indicates that barley husks work as a strong barrier against enzymatic diffusion of both amylase and glycosidase. Large particles have been shown to survive ruminal attack and pass to small intestine for digestion (Owens *et al.*, 1986).

In conclusion, microscopic examinations for in vitro digested milled barley fragments differ in particle size and this indicates that the extent of starch digestion is dependent on fragment particle size (i.e., heterogeneity of particle size distribution). SEM for whole barley grain revealed that the presence of barley husk prevents any enzymatic diffusion and thus no starch digestion takes place.

References

Adrados BP, Galbe M and Zacchi, G. 2005. Pretreatment of barley husk for bioethanol production. *J Chem Technol Biotechnol.*, **80**:85–91.

Al-Rabadi GJ. 2012. Influence of hammer mill screen size on processing parameters and starch enrichment in milled sorghum. *Cereal Res Comm.*, DOI:10.1556/CRC.2013.0016.

Al-Rabadi GJ. 2013. Effect of hammer mill screen size on processing parameters and starch enrichment in milled barley. *Jordan J Agri Sci*., **9:**162-168.

Al-Rabadi GJ, Gilbert R and Gidley M. 2009. Effect of particle size on kinetics of starch digestion in milled barley and sorghum grains by porcine alpha-amylase. *J Cereal Sci.*, **50** (2): 198-204.

Al-Rabadi GJ, Torley P, Williams BA, Bryden WL and GidleyM.J. 2012. Particle size heterogeneity in milled barley and sorghum grains: Effects on physico-chemical properties and starch digestibility. *J Cereal Sci*., **56**: 396-403.

Amerah AM, Ravindran V, Lentle RG and Thomas DG. 2007. Feed particle size: Implications on the digestion and performance of poultry. *World Poultry Sci J*., **63:** 439-455.

Audet L. 1995. Emerging feed mill technology: keeping competitive. *Animal Feed Sci Technol*., **53**: 157-170.

Douglas JH, Sullivan TW, Bond PL, Struwe FJ, Baier JG and Robeson LG. 1990. Influence of grinding, rolling, and pelleting on the nutritional-value of grain sorghums and yellow corn for broilers. *Poultry Sci.*, **69**: 2150-2156.

Franco CML, Preto, SJR and Ciacco CF. 1992. Factor that affect the enzymatic degradation of natural starch granules-effect of the size of granules. *Starch/ Stärke*. **44**: 422-426.

Helbert W, Schu lein M and Henrissat B. 1996. Electron microscopic investigation of the diffusion of *Bacillus licheniformis* α-amylase into corn starch granules. *Inter J Biol Macromolecules*, **19**: 165-169.

Kim K. 2002. Hammer mills or roller mills. Feed Manufacturing (MF-2048). Manhattan, Kansas State University.

Matsubara T, Ammar YT, Anindyawati S, Yamamoto K, Ito Mand MN. 2004. Degradation of raw starch granules by α-amylase purified from culture of *Aspergillus awamori* KT-11. *J Biochem Molecular Biol*., **37**: 422-428

Owens FN, Zinn RA and Kim YK. 1986. Limits to starch digestion in the ruminant small intestine. *J Animal Sci*., **63**: 1634-1648.

Robertson HG, Wong DWS, Lee CC, Wagschal K, Smith MR and Orts WJ. 2006. Native or raw starch digestion: a key step in energy efficient biorefining of grains. *J Agri Food Chem*., **54**:353-365.

Rooney LW and Pflugfelder RL. 1986. Factors affecting starch digestibility with specialemphasis on sorghum and corn. *J Animal Sci.,* **63**: 1607-1623.

Seerley RW, Vandergrift WL and Hale OM. 1988. Effect of particle size of wheat on performance of nursery, growing and finishing pigs. *J Animal Sci.,* **66**: 2484-2489

Srikaeo K. 2008. Microscopy and image analysis techniques for quality control in food industry: A case study of wheat grain cooking process. *J Microscopy Soc Thailand.* **22**: 46-49.

Srikaeo K, Furst JE, Ashton JF and Hosken RW. 2006. Microstructural changes of starch in cooked wheat grains as affected by cooking temperatures and times. *LWT*. 39: 528-533.

Sun Z and Henson A. 1990. Degradation of native starch granules by barley glucosidases. *Plant Physiol*., **94**: 320-327

Svihus B, Uhlen AK and Harstad OM. 2005. Effect of starch granule structure, associated components and processing on nutritive value of cereal starch: A review. *Animal Feed Sci Technol*., **122**: 303-320.

Szczodrak J and Pomeranz, Y. 1991. Starch and enzyme resistance starch from high amylose barley starch. *Cereal Chem.*, **68**: 589-596.

Tester RF, Karkalas J and Qi X. 2004. Starch structure and digestibility enzyme-substrat relationship . *World's Poultry Sci Asso*., **60**: 186-195.

Wondra K, Hancock J, Behnke K and Stark C. 1995. Effects of mill type and particle size uniformity on growth performance, nutrient digestibility, and stomach morphology in finishing pigs. *J Animal Sci*., **73** (9): 2564-2573.

JJBS

Volume 7, Number 3, September .2014
ISSN 1995-6673
Pages 205 - 209

Jordan Journal of Biological Sciences

Evaluation of Antioxidant, Antimicrobial and Cytotoxicity of *Alcea kurdica* Alef

Suhailah W. Qader[1,*] and Hassan M. Awad[2]

[1]*Department of Biology-College of Education, Scientific Departments, University of Salahaddin- Hawler 44001, KRG, Iraq;*

[2]*Institute of Bioproduct Development, Universiti Teknologi Malaysia, UTM Skudai, Johor 81310, Malaysia*

Received: May 17, 2014 Revised: June 23, 2014 Accepted: July 1, 2014

Abstract

The purpose of this study is to evaluate the aqueous extract of *Alcea kurdica* Alef for antioxidant and antimicrobial activity as well as potential toxicity. Antioxidant activities were evaluated using 1,1-diphenyl-2- picrylhydrazyl (DPPH) free radical scavenging and Ferric Reducing Antioxidant Power (FRAP) reducing capacity assays as well as total phenolic compounds (TPC). Antimicrobial activity was assessed against some Gram-positive and Gram-negative bacteria, yeasts and filamentous fungi using the agar dilution method. The plant extract was also assessed for *in-vitro* toxicity using the Promega Cell Titer 96 AQueous Non-Radioactive Cell Proliferation (MTS) assay. The aqueous extract of *A. kurdica* demonstrated potent free radical scavenging activity of 64% ± 1.64% and ferric reduction capacity of 2955.0 ± 0.04 mmol/g, as well as having 88.0 ± 0.002 mg gallic acid equivalents /gram plant extract. The aqueous extract of *A. kurdica* had modest antimicrobial activity against most tested microorganisms. Moreover, the plant extract did not have any toxic effects on human lung fibroblasts. Based on these findings, we conclude that *A. kurdica* may be utilized to prevent the growth of some microorganisms. The plant constituents behind these effects are the antioxidants and phenols. Further studies are needed to evaluate the therapeutic utility of *A. kurdica* extracts as antimicrobial agents.

Keywords: *Alcea kurdica* Alef, Antioxidant, Antimicrobial activity, Cytotoxicity.

1. Introduction

Recently, scientists have paid more attention to the role of natural antioxidants, mainly phenolic compounds, which may have more antioxidant activity than synthetic antioxidants (Velasco and Williams, 2011). Natural products, particularly those present in medicinal plants, have gained more interest as food ingredients because of their safety, accessibility, and positive impact on health (Ebrahimabadi *et al.*, 2010). A variety of natural antioxidant compound purified and derived from plant resources have been demonstrated to scavenge free radicals (Loo *et al.*, 2007). Epidemiological studies suggest that the consumption of plants can protect humans against oxidative damage by inhibiting or scavenging free radicals and reactive oxygen species (ROS) (Sun *et al.*, 2002; Materska and Perucka, 2005). Natural antioxidants exhibit a wide range of pharmacological activities, and have shown anticancer, anti-inflammatory, anti-aging, anti-ulcer, and antimicrobial properties (Mayne, 2003; Pinnell, 2003). For the past several decades, a variety of vegetables, crops, spices, and medicinal herbs have been analyzed in an effort to identify new and potentially useful antioxidants (Zheng and Wang, 2001). It has become evident that natural products may reduce oxidative stress through antioxidant action. For example, various phenolics and flavonoids, which are found naturally in fruit, vegetables, and some beverages, have been demonstrated to exert antioxidant effects through a number of different mechanisms (Nijveldt *et al.*, 2001).

Alcea kurdica, which belongs to the family Malvaceae, is a very polymorphic and widespread species found in east Iraq and west Iran. *Alcea* is an important source of mucilage and are widely distributed with about 70 species (Pakravan and Ghahreman, 2003). *Alcea* are usually found in the Penjween, Sharbazher area, and in Haji Omran and Garaguin in Kurdistan of Iraq. The *Alcea* is a delicate plant having sharp, pale yellow, dark reddish stems, and greyish green foliage. Traditionally *A. kurdica* have been widely used among the Kurdish population to treat a variety of diseases including tonsillitis, gastric ulcers, duodenal ulcers, pneumonia, urinary tract infections, and alopecia (Mati and de Boer, 2011). Based on literature survey, there are no ethnopharmacological studies on *A. kurdica* that originate from Kurdistan of

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

Iraq. Based on the importance of *A. kurdica* in medicinal folk, this study was conducted to evaluate the antioxidant and antimicrobial activities of *A. kurdica* as well as potential toxicity against the human normal lung fibroblast cell line (Hs888Lu).

2. Materials and Methods

2.1. Plant Extract

The dried leaves of the *A. kurdica* plant were purchased at the herbal market, Erbil, Iraq. The Voucher-ID and Vernacular name of *A. kurdica* were identified as Alef. EM2.1 Malvaceae Gule hero (Mati and de Boer, 2011). After identification, the plant leaves were ground into powder using an electrical blender then extracted using water solvent at a ratio of 1:20. The mixture was heated and stirred on a hotplate for 3 hrs at 65° C followed by cooling and filtration using Whatman No. 1 filter paper and a filter funnel. The mixture was evaporated under reduced pressure in Eyela™ rotary evaporator (Sigma-Aldrich, USA) and subjected to lyophilisation by freeze-drying (Labconco, Kansas, USA) to produce a powdered form of the extract. The extract was stored at -20° C for later use.

2.2. Antioxidant Experiments

The antioxidant activity of the aqueous extract was determined using the 1,1-diphenyl-2- picrylhydrazyl (DPPH) radical assay which is based on the transfer of electrons between the DPPH reagent and the plant extracts. The method described by (Loo *et al*., 2007; Erel, 2004; Gorinstein *et al.*, 2003) was utilized with minor modifications. The DPPH value was expressed as percentage scavenging of the DPPH based on the following equation: DPPH (%) = (Absorbance of blank – Absorbance of sample) x 100 / Absorbance of sample. Each test was performed three times in triplicate and the results were expressed as mean percentage. Ferric Reducing Antioxidant Power (FRAP) assay was performed according to a previously described method (Erel, 2004). FRAP values were expressed as mmol of ferric reducing activity of the plant extract per gram of dry weight and Ferrous sulfate heptahydrate (FeSO4x7H2O) was used as a standard, based on the following equation: FRAP value of sample in mmol/L = (change in absorbance of sample from 0 to 4 min / change in absorbance of standard from 0 to 4 min) x FRAP value of standard.

2.3. Total Phenolic Compounds (TPC)

The total phenolic compounds (TPC) of aqueous plant extracts were determined by the Folin-Ciocalteu method (Miliauskas *et al.*, 2004), using gallic acid as a standard. Total phenolic content of the samples was determined, and the amounts of phenolic compounds in plant extracts were expressed in mg/g of extract and gallic acid equivalents (GAE), respectively. Each test was carried out three times in triplicates. Values were expressed as means.

2.4. Antimicrobial Activity Experiment

The ability to inhibit the growth of Gram-positive and Gram-negative bacteria, yeasts and filamentous fungi was observed using an overlay method (Williams *et al*., 1993). Antibacterial screening utilized the agar dilution method as previously described (Afolayan and Meyer, 1997). The dried plant extracts were dissolved in methanol to a final concentration of 50 mg/mL and sterilized by filtration through 0.45 µm Millipore filters. The activity of this solution was compared with different standard antimicrobial agents. Discs of streptomycin (S, 10 µg) and tetracyciline (TE, 30 µg) were used as positive controls for bacteria, neomycin (N, 30 µg) and nystatin (NY, 100 µg) were used as positive controls for fungi. Sterilized paper discs without the extract or antimicrobial agents were used as negative controls for all microorganisms. Final dilutions of the extract (ranging between 0.1 and 10 mg/mL) were prepared in molten Müeller Hinton agar medium (Lab M., Bury, Lancashire, UK) maintained in a water bath at 50° C. The organisms were streaked in radial patterns on the agar surface. Plates were incubated under aerobic conditions at 37° C for 24 hrs for the bacteria or 28° C for 48 hrs for the fungi. The organisms used were: *Bacillus subtitles*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Enterobacter aerogenes*, *Candida albicans*, *Salmonella enterica*, *Fusarium oxysporum*, *Cladosporium macrocarpum*, and *Fusarium solani*. The bacteria were slanted on nutrient agar (Merck, Darmstadt, Germany), the yeast was slanted on Sabaroud's agar medium (Lab M., Bury, Lancashire, UK), and the fungi were slanted on potato dextrose agar medium (Lab M Limited, Bury, Lancashire, UK). Each test was conducted in triplicate. The lack of visible growth on the agar plates was used to indicate the inhibitory activity of the extracts.

2.5. MTS Assay

The cytotoxic activity of aqueous extract of *A. kurdica* was determined using Promega Cell Titer 96 AQueous Non-Radioactive Cell Proliferation (MTS) assay (Lestari et al., 2005). The MTS assay is a colorimetric test for the determination of cell viability in cytotoxicity assays. The assay utilized the human normal lung fibroblast cell line (Hs888Lu), purchased from American Type Culture Collection (ATCC, The Global Bioresource Centre, Manassas, VA, USA). Hs888Lu cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Sigma, USA) with high glucose content, 1% non-essential amino acids (PAA Laboratory GmbH, Austria), 2% L-glutamine (200 mM) (Sigma, USA), 1% penicillin/streptomycin (100 x) (PAA Laboratories GmbH, Austria), 1% sodium pyruvate (1 mM) (Sigma-Aldrich, USA), and supplemented with 10% fetal bovine serum (FBS, PAA Laboratory GmbH, Austria). Cells (1 x 105 cells/mL) were seeded in a 96-well plate using 100 µL per well, and incubated at 37° C with 5% CO2 in a humidified atmosphere for 24 hrs before the addition of the plant extract. Dilutions of *A. kurdica* extract ranging in concentration from 125 µg/mL to 2000 µg/mL were added to the culture plate in triplicates, and then incubated for 24 hrs under the same conditions. Following the treatment, 20 µL of the MTS reagent (pre-warmed to 37° C) was added to each of the 96-wells and the plate was incubated at 37° C for 3 hrs. The absorbance was recorded using Glomax multi detection system (Promega, USA) at 492 nm. The experiment was conducted three times in

triplicates and the mean was calculated and expressed as percentage of the value observed with no plant extract treatment (control).

2.6. Statistical Analysis

The data were analyzed using One-way ANOVA test by Statistical Package for Social Sciences (SPSS) version 17.0 program. A *p* value less than 0.05 was considered statistically significant.

3. Results and Discussion

3.1. Antioxidant Experiments

The DPPH free radical scavenging abilities of the positive control and plant extracts are expressed as a percentage of inhibition. Based on the values calculated from the linear standard curves (y = 2.002x; R2 = 0.9819) as in Table 1, the aqueous extract of *A. kurdica* showed high free radical scavenging activity towards DPPH with 69.4% ± 1.19% inhibition. The ferric reducing ability of A. kurdica was 258.33 ± 0.97 mmol/g (Table 1). The FRAP value was calculated from a standard calibration curve equation (y = 0.0011x; R2 = 0.9987) as in Table 1. The TPC of *A. kurdica* was 80 ± 0.98 mg gallic acid equivalents per g of extract (Table 1). The potent radical scavenging effect is positively associated with the high content of phenolic components consistent to what has been previously reported by Gorinstein and coworkers (Gorinstein *et al*., 2003; Qader *et al*., 2011). Furthermore, Scalzo and coworkers (Scalzo, 2005) and Giorgi and coworkers (Giorgi *et al.*, 2005) have demonstrated a correlation between antioxidant activity and TPC. Therefore, the antioxidant capacity of A. kurdica could be related to its phenolic content.

Table 1. The antioxidant activity and total phenolic compounds of aqueous extract of A. kurdica

Antioxidant assay	Gallic Acid	Vitamin C	Quercetin	*A. kurdica*	Standard Curve Equation
DPPH (%)	88.8 ± 0.41	87.5 ± 0.02	-	69.4± 1.19	y = 2.002x R^2 = 0.9819
FRAP (mmol.g^{-1})	1216.67 ± 1.02	432.67 ± 0.14	-	258.33 ± 0.97	y = 0.0011x R^2 = 0.9987
TPC (mg GA eq. g^{-1})	-	-	118± 0.37	80 ± 0.98	y = 0.9917x R^2 = 0.9984

DPPH % and FRAP (mmol.g^{-1}) values represent the mean ± SEM of triplicate experiments. "-": not used.

3.2. Antimicrobial Activity of A. kurdica Extract

The aqueous extracts of *A. kurdica* exhibited modest antimicobial activity against different microbial organisms (Table 2). The extract did not inhibit the growth of *Staphylococcus aureus*, *Bacillus subtilis*, *Enterobacter aerogenes*, *Salmonella enterica*, and *Fusarium solani*. On the other hand, it demonstrated modest antimicrobial activity against *Escherichia coli*, *Pseudomonas aeruginosa*, *Candida albicans*, *Cladosporium macrocarpum*, and *Fusarium oxysporium*. Among the tested microorganisms, the filamentous fungi *Cladosporium macrocarpum* and *Fusarium oxysporium*, were the most affected by the extract, with inhibition zones of 15 mm. Therefore, the *A. kurdica* plant is a potential source of novel antimicrobial compounds especially against some fungal and bacterial pathogens (Gram negative). The antimicrobial activity of this plant extract has been previously reviewed by Sharifi (2012). Generally, phenolic compounds have been illustrated to possess different bioactivities including free radical scavenging. Furthermore, the antioxidant activities of phenolics provide important protective mechanisms in a variety of disease conditions (Alshawsh *et al.*, 2012). Several studies have reported that phenolic compounds mediate the antimicrobial activities of various plant extracts (Dordevic *et al.*, 2007; Alshawsh *et al.*, 2012; Ebrahimabadi *et al.*, 2010). Hence, this study has evaluated *A. kurdica*'s antioxidant and phenolic contents as well as its antimicrobial activity.

Table 2. The antimicrobial activity of *A. kurdica* aqueous extract

Organisms	Bacteria						Fungi			
	Gram Positive		Gram Negative				Unice-llular	Filamentous		
	B.s	S.a	*E.a*	S. e	P.a	E.c	C.a	C.m	F.o	F.s
Inhibition Zone (mm)										
AKE	00	00	00	00	12	12	13	15	15	00
S 10µg	14	ND	ND	ND	ND	12	00	ND	ND	00
TE 30µg	18	ND	ND	ND	ND	23	00	ND	ND	00
N 30 µg	0	ND	ND	ND	ND	00	16	ND	ND	15
NY 100µg	0	ND	ND	ND	ND	00	00	ND	ND	15

AKE *A. kurdica* extract, *B.s*: *Bacillus subtilis*. *S.a*: *Staphylococcus aureus*. *E.a*: *Enterobacter aerogenes*. *S.e*: Salmonella enterica. P.a: *Pseudomonas aeruginosa*. *E.*c: *Escherichia coli)*. *C.a*: *Candida albicans*. *C.m*: Cladosporium macrocarpum. F.o: Fusarium oxysporum. and F.s: Fusarium solani. 00 = no inhibition zone. ND = not detected, S: streptomycin. TE: tetracycline (TE), N: neomycin, NY: nystatin.

3.3. MTS Assay

In this study, normal lung fibroblast cells (Hs888Lu) have been used for toxicity evaluation. The results of cytotoxic activity of aqueous extract of *A. kurdica* are summarized in Figure 1. Data were expressed as percentage of the value observed with no plant treatment (control). Cytotoxicity has been assessed using different concentrations of the extract. None of the extract concentrations had any cytotoxic effect as there were no significant differences ($P<0.05$) between the cytotoxicity of the plant extract concentrations and the control. Previous studies have used normal lung cells as a model to evaluate toxicity (Najim *et al.*, 2010). This is the first time that the cytotoxic activity of *A. Kurdica* has been assessed against the human normal lung fibroblast cell line Hs888Lu.

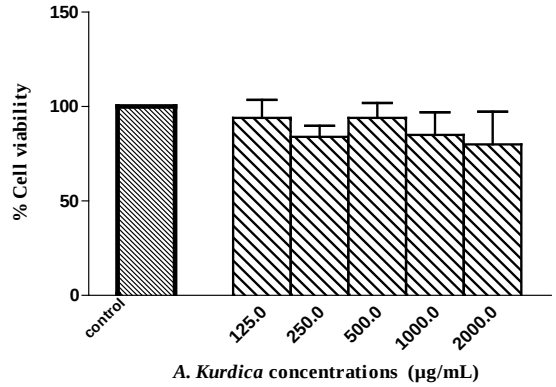

Figure 1. Cytotoxic activities of aqueous of *A. kurdica* in normal lung fibroblast Hs888Lu cell line at concentrations of 125-2000 µg/mL and 24 hrs exposure times. Each bar represents the mean ± SEM of triplicates. Control: no plant extract treatment.

4. Conclusion

In the present study, *A. kurdica* exhibited potent antioxidant activity which might be useful for the therapy or management of disorders involving ROS-mediated pathology. Further *A. kurdica* demonstrated interesting antimicrobial activity against growth of selected microorganisms. Notably, the plant extract did not have any cytotoxic effects against the normal lung fibroblast (Hs888Lu) cell line. Ultimately, this study confirmed that the aqueous extract of *A. kurdica* is able to scavenge free radicals and possessed modest antimicrobial activity.

Acknowledgments

The authors thank the Department of Biology, College of Education, University of Salahaddin and Institute of Bioproduct Development, Universiti Teknologi Malaysia, Chemistry of Natural and Microbial product Dept., Pharmaceutical industry Dev, and the National Research Centre, El-Bohouth Street, Dokki, Cairo, Egypt for their support.

Conflict of Interest

The authors declare no conflicts of interest.

References

Afolayan AJ and Meyer J J M. 1997. The antimicrobial activity of 3, 5, 7-trihydroxyflavoneisolated from the shoot of *Helichrysum aureonitens*. *J. Ethnopharmacol.* , **57**(3): 177-181.

Alshawsh M A, Abdulla M A, Ismail S, Amin Z A, Qader S W, Hadi H A and Harmal N S. 2012. Free radical scavenging, antimicrobial and immunomodulatory activities of *Orthosiphon stamineus*. *Molecules*, **17**(5): 5385-5395.

Dordevic S, Petrovic S, Dobric S, Milenkovic M, Vucicevic D, Zizic S and Kukic J. 2007. Antimicrobial, anti-inflammatory, anti-ulcer and antioxidant activities of *Carlina acanthifolia* root essential oil. *J Ethnopharmacol.*, **109**(3): 458-463.

Ebrahimabadi A H, Mazoochi A, Kashi F J, Djafari-Bidgoli Z and Batooli H. 2010. Essential oil composition and antioxidant and antimicrobial properties of the aerial parts of *Salvia eremophila* Boiss. from Iran. *Food and Chemical Toxicol.*, **48**(5): 1371-1376.

Erel O. 2004. A novel automated direct measurement method for total antioxidant capacity using a new generation, more stable ABTS radical cation. *Clinical Biochem.*, **37**(4): 277-285.

Giorgi M, Capocasa F, Scalzo J, Murri G, Battino M and Mezzetti B. 2005. The rootstock effects on plant adaptability, production, fruit quality, and nutrition in the peach. *Scientia Horticulturae*, **107**(1): 36-42.

Gorinstein S, Martin-Belloso O, Katrich E and Lojek A. 2003. Comparison of the contents of the main biochemical compounds and the antioxidant activity of some Spanish olive oils as determined by four different radical scavenging tests. *J Nutritional Biochem.*, **14**(3): 154-159.

Lestari F, Hayes A, Green A and Markovic B. 2005. *In vitro* cytotoxicity of selected chemicals commonly produced during fire combustion using human cell lines. *Toxicol in vitro*, **19**(5): 653-663.

Loo A, Jain K and Darah I. 2007. Antioxidant and radical scavenging activities of the pyroligneous acid from a mangrove plant, *Rhizophora apiculata*. *Food Chem.*, **104**(1): 300-307.

Materska M and Perucka I. 2005. Antioxidant activity of the main phenolic compounds isolated from hot pepper fruit (*Capsicum annuum* L.). *J Agricultural Food chem.*, **53**(5): 1750-1756.

Mati E and de Boer H. 2011. Ethnobotany and trade of medicinal plants in the Qaysari Market, Kurdish Autonomous Region, Iraq. *J Ethnopharmacol.*, **133**(2): 490-510.

Mayne S T. 2003. Antioxidant nutrients and chronic disease: use of biomarkers of exposure and oxidative stress status in epidemiologic research. *J Nutrition*, **133**(3): 933S-940S.

Miliauskas G, Venskutonis P and Van Beek T. 2004. Screening of radical scavenging activity of some medicinal and aromatic plant extracts. *Food Chem.*, **85**(2): 231-237.

Najim N, Bathich Y, Zain M M, Hamzah A S and Shaameri Z. 2010. Evaluation of the bioactivity of novel spiroisoxazoline type compounds against normal and cancer cell lines. *Molecules*, **15**(12): 9340-9353.

Nijveldt R J, van Nood E, van Hoorn D E C, Boelens P G, van Norren K and van Leeuwen P A M. 2001. Flavonoids: a review of probable mechanisms of action and potential applications. *American J Clin Nutrition*, **74**(4): 418-425.

Pakravan M and Ghahreman A. 2003. Some new combinations and synonyms in *Alcea* (Malvaceae) from Iran. *Ann Naturhist Mus Wien.,* **104B:** 713-716.

Pinnell S R. 2003. Cutaneous photodamage, oxidative stress, and topical antioxidant protection. *JAmerican Academy of Dermatol.,* **48**(1): 1-18.

Qader S W, Abdulla M A, Chua L S, Najim N, Zain M M and Hamdan S. 2011. Antioxidant, total phenolic content and cytotoxicity evaluation of selected Malaysian plants. *Molecules,* **16**(4): 3433-3443.

Scalzo J, Politi A, Pellegrini N, Mezzetti B and Battino M. 2005. Plant genotype affects total antioxidant capacity and phenolic contents in fruit. *Nutrition,* **21**(2): 207-213.

.

Sun J, Chu Y F, Wu X and Liu R H. 2002. Antioxidant and antiproliferative activities of common fruits. *J Agricultural Food Chem.,* **50**(25): 7449-7454.

Velasco V and Williams P. 2011. Improving meat quality through natural antioxidants. *Chilean J Agricultural Res.,* **71**(2): 313-322.

Williams S T, Goodfellow M, Alderson G, Wellington E M H, Sneath P H A and Sackin M J. 1993. Numerical classification of *Streptomyces* and related genera. *J Gen Microbiol.,* **129**: 1743–1813.

Zheng W and Wang S Y. 2001. Antioxidant activity and phenolic compounds in selected herbs. *J Agricultural Food Chem.,* **49**(11): 5165-5170

JJBS

Volume 7, Number 3, September .2014
ISSN 1995-6673
Pages 211 - 215

Jordan Journal of Biological Sciences

Mating Frequency, Duration and Time in Baluchistan Melon Fly *Myiopardalis pardalina* (Bigot) (Diptera: Tephritidae)

Morteza Movahedi Fazel [1,*] and Ali Mohammadipour[2]

[1] *Department of Plant Protection, Agricultural College, ZanjanUniversity, Zanjan,*

[2] *Department of Entomology, Institute of Plant Protection, Tehran,Iran*

Received: May 21, 2014 Revised: June 21, 2014 Accepted: July 4, 2014

Abstract

Myiopardalis pardalina (Bigot) (Diptera: Tephritidae) is one of the injurious pests that damage melon fruits. The male sterility technique is one of the genetic methods that have been proposed for controlling fruit flies. This method is more effective in once-mated females. So mating frequency, starting time and mating duration by females and males of Baluchistan melon fly were studied in the laboratory (Department of Entomology, Institute of Plant Protection, Tehran, Iran). Mean number of matings/female was 5.83±0.48 during the 8 day test period. The presence or absence of the host did not have any significant influence on mating frequency. The mean number of matings/male was 6.26±0.45. Mating predominantly occurred in early afternoon. Mating duration in female and male series was 4.95±0.598 and 6.822±0.378 hours, respectively. The first mating usually took longer in comparison with other matings.

Keywords: Mating frequency, Baluchistan melon fly, *Myiopardalis pardalina*, Mating duration, Mating time

1. Introduction

Insect reproduction involves two behaviors: mating and oviposition (Jimenez-Perez and Wang, 2003). In general, copulation is assumed to be costly for many reasons (Hunter *et al.,* 1993). Females of many diverse animal species mate multiple times in nature (Andersson, 1994; Johnson and Burley, 1997). Such multiple mating is performed with different male partners as in the fruit fly, *Drosophila melanogaster* (Fuerst *et al.,* 1973). However, sometimes females remate with the same male partner (repeated mating) (Hunter *et al.,* 1993). Repeated mating is only reported for a limited number of species (Petrie, 1992; Petrie *et al*., 1992; Hunter *et al.,* 1993; Choe, 1995; Lens *et al.,* 1997; Andrade and Mason, 2000).

The frequency of mating in Tephritid fruit flies is an important aspect of their sexual behavior. It is relevant to the development of those pest control programs based in part on sexual interactions. For example, sex attractants developed for females may be more effective for species that remate frequently and may then repeatedly respond to male sex pheromone (Landolt, 1994). A multiple mating may increase the predation risks associated with searching for and mating with males, either because females have to search in risky areas(Koga *et al.,* 1998) or because during mating vigilance and mobility are reduced (Jennions and Petrie, 2000). Females receiving multiple male contributions lay more eggs (Ridley, 1988) and often larger ones (Fox, 1993) than do once-mated females, indicating a large effect of male derived nutrients on females reproduction (Fox *et al.,* 1995). A number of hypotheses have been proposed to explain the occurrence of multiple mating, and there is a general empirical support for these (reviewed in Petrie *et al.,* 1992).

In many insects, females are receptive for much of their adults' life and so mate more than once. However, the evolution of patterns of female receptivity leading to multiple mating in short-lived animals is something of a mystery because the cost to females of mating more than once (increased risk of predation, time lost from feeding and oviposition) usually appear to out weight the benefits (Thornhill and Alcock, 1983; Jennions, 1997).

The potential or hypothesized benefits for females of multiple mating fall into two general classes: material benefits and/or genetic benefits (Reynolds, 1996). In general, material benefits enhance female fitness directly through increased numbers or size of eggs, whereas genetic benefits enhance female fitness indirectly through increased genetic quality of offspring (Zeh and Zeh, 1996). Material benefits may include nutritional resources from nuptial gift from males (Gwynne, 1997; Eberhard, 1996), a reduction in male harassment (Rubenstein, 1984; Arnqvist, 1989), and replenishment of sperms if one

* Corresponding author. e-mail: movahedi@znu.ac.ir.

mating provides insufficient sperms to fertilize all the eggs a female which may produce in her lifetime (Thornhill and Alcock, 1983). Alternatively, genetic benefits of multiple mating may include opportunities to manipulate offspring paternity (Birkhead and Mollar, 1992; Ridley, 1993), and to avoid inbreeding (Brooker *et al*., 1990; Madsen *et al.*, 1992). Also, it can decrease the chances of fertilization by sperm that are genetically defective due to their age (Halliday and Arnolds, 1987) or incompatible genotype (Zeh and Zeh, 1996). In some lepidopteran species the number of apyrene sperm in the spermatheca may influence female remating (He *et al*., 1995).

The mating frequency varies among females of species of fruit infesting tephritids. Females of the apple maggot fly, *Rhagoletis pomonella* (Walsh) mate frequently, as often as weekly, possibly to maintain fertility levels (Prokopy and Roitberg, 1984). The Mediterranean fruit fly, *Ceratitis capitata* Wiedemann (Nakagawa *et al*., 1971), and *Anastrepha suspense* (Loew) (Sivinski and Heath, 1988), are thought to mate usually once, with rematings due either insemination failures at first mating or to sperm depletion following extensive oviposition. In papaya fruit fly, *Toxotrypana curvicauda* Gerstaecker, the most mature females mated more than once when held with immature papaya fruit (Landolt, 1994). The Baluchistan melon fruit fly, *Myiopardalis pardalina* is one of the important pests in Iran that attack melon varieties and peppo. The mean damage annually reaches 30-80% on melon varieties in Iran (Sirjanii, 1995; Eppo, 2013). However this study aimed at investigating the mating frequency of *M. pardalina*, in order to gather basic information needed for evaluating the possibility of using the male sterility technique or sex attractants for management and control of this pest.

2. Materials and Methods

Baluchistan melon fruit flies were obtained as mature larvae (3rd instar) from infested melon fruit collected from open fields in the Varamin area of Tehran province-Iran. After getting out of fruit, the larvae were pupated in potting soil. Pupae were screened and transferred to special cages for adult emergence. Newly emerged adult flies were daily sorted by sex (females have an obvious ovipositor) and kept in separate cages and fed with a solution of sucrose: protein hydrolysate in (5:1 w/w) ratio. The cages were kept under laboratory conditions of 28±2^{o}C temperatures, 50±10% RH, and natural light conditions during July till August, nearly 16 L: 8 D h. The authors can do this easily.

Mating frequency was determined in the following series: A) mature female held with green immature melon (2-4 cm in diameter and 5-8 cm in length, as a preferred oviposition site (Sirjanii, 1995)), B) mature females without fruit, C) males without fruit, and D) paired females and males with fruit. In series (A), reproductively mature females (3-day-old) (Sirjanii, 1995) were separately kept per cage from 08:00 to 20:00 hours every day for a period of 8 days. Each morning at 08:30, a male (virgin and the same age as the female) was kept in each cage and observations were made at 30-min- interval until 20:00 hs, then the male was removed (if the mating was finished). Because matings on average take 264 min (Sirjanii, 1995), it was assumed that none would be missed if observations were made every 30 min. At each observation time, mating recorded and oviposition monitored of females held with fruit. All the matings that lasted less than 30 min were assumed to be followed by rejection and omitted from the results. After flies mated, the male was replaced. Twenty-five females were separately held with males and melon fruit within the cages that included a small cup of sugar and water on cotton. In the (B) series, the same number of females was separately held with males, without melon fruit. During the experiments conducted to determine mating frequency of females without fruit, melon was also excluded from the laboratory to avoid the host odor effects on mating behavior. In series (D) the mating frequency of a given pair with fruit was observed during the 8-day period in thirty-five series to determine the occurrence of repeated matings with the same male. In this experiment, the male was not excluded from the cage throughout the test. To assess mating frequency of males (series C), a similar protocol was followed. For eight consecutive days, at 08:00 hours, a mature virgin female was kept in each cage containing one male. Observations were made each 30 min, until 20:00 hs, when the female was removed (if mating was finished). The female was replaced after each copulation, with another mature virgin female. This was done for 17 mature individuals (≥3 days old). All the males were held without melon fruit. In addition, the time when mating began, copulation duration and the daily rhythm of mating (dN/dt) were also recorded and differences between various series were compared. The daily rhythm of mating was determined by dividing the number of matings that happened in all of replicates during each age to number of replicates.

2.1. Statistical Analysis

Mean mating frequencies for all the series were compared using Student t-test (Gomez and Gomez, 1984). Also, the mating frequencies, the time of mating initiation, copulation duration and dN/dt were compared in all the series using Duncan's Multiple Range test (SAS 9.1, SAS Institute, Inc). The data were analyzed at a probability level of 0.05.

3. Results

In series A, females, held in cages with melon fruit, mated more than 5 times, with most females mating more than once. Mean (±SE) number of matings/female was 5.83±0.48 (n=25) during the 8-day test period. In series B, all females, held in cages without fruit, showed multiple mating. Mean number of matings/female was 5.36±0.39 (n=25) during the test period. There were no significant differences between series A and B ($P > 0.05$). In series C, males mated more than six times over the course of the 8-day test period, with nearly all mating multiple times. Mean number of matings per male was 6.29±0.45 (n=17). Mean number of matings in series (D) (remating with the same male) was 5.63±0.38 (n=35), which did not reveal any significant difference with series (B) and (C). Also, the daily mean mating frequency (dN/dt) was determined

(Table 1). These results showed a significant decrease of dN/dt for the 8-9 days old females in (A) series ($P < 0.01$). Matings by females in different test series were predominant during the early part of afternoon (14-18 hs) (Figure 1) and showed a significant difference with the other observational periods ($P < 0.01$). This time showed some variations based on different times of mating (Table 2). The mean mating duration in series A and C were 4.95±0.598 and 6.822±0.378 hs, respectively, and showed significant differences between series based on history of mating (Table 3). The first mating in series A showed longer duration and other arrangements showed significantly irregular fluctuations. Longer mating duration observed in second mating in series C. The duration of first and third mating in series A and C were similar but in other matings the copulation duration in series A was shorter than that in series C. Also it's revealed that the initial mating in each series lasted more than the final mating (Table 3).

Table 1. Daily mean(±SEM) mating frequency(dN/dt) in relation to adult age in *M. pardalina*.(N=10).

Age(days)	Series A*	Series C	Series D
3	1±0.0001a	0.9±0.1a	0.9±0.1a
4	0.7±0.15b	0.8±0.13a	1±0.0001a
5	0.8±0.13ab	0.9±0.1a	0.9±0.1a
6	0.7±0.15b	0.9±0.1a	0.8±0.13ab
7	0.9±0.1a	1±0.0001a	0.8±0.13ab
8	0.2±0.13c	1±0.0001a	1±0.0001a
9	0.2±0.13c	1±0.0001a	0.6±0.16b

Series A: mature female held with fruit for determination of multiple mating in females , *Series C*: mature males without fruit , for determination of multiple mating in males , *Series D*: same pair for determination of repeated mating. * Means within columns followed by the same lower-case letter are not significantly different at the 5% level by Duncan's multiple range test.

Table 2. The mean (± SE)(n) of starting time(h) of copulation in different series based on mating history in *M. pardalina.*

Mating arrangement	Series A*	Series C	Series D
1St	14.27±0.54 (18)b	15.14±0.6 7 (30)a	14.61±0.36 (28)a
2nd	16.38±0.4 (15)ab	12.94±0.8 1 (28)a	15.07±0.32 (28)a
3rd	15.57±0.44 (18) ab	13.56±0.5 5 (22)a	15.4±0.36 (25)a
4th	17.16±0.52 (15)ab	14.5±0.51 (15)a	15.83±0.43 (23)a
5th	17.63±0.55 (16)a	14.78±0.4 8 (14)a	16.22±0.3 (23)a
6th	18.83±1.3 (15)a	15.03±0.6 1 (15)a	16.85±0.4 (23)a

Series A: mature female held with fruit for determination of multiple mating in females *Series C*: mature males without fruit, for determination of multiple mating in males *Series D*: same pair for determination of repeated mating *: Means within columns followed by the same lower-case letter are not significantly different at the 5% level by Duncan's multiple range test.

Table 3. The mean (±SE) (n) of mating duration in different series based on mating arrangement in *M. pardalina*.

Mating arrangement	Series A (Females) M ± SE	Series C (males) M ± SE
1st	7.383 ± 0.51 (30) (a)A*	6.861 ± 0.66 (18) (a) AB
2nd	3.768 ± 0.49 (28) (a)C	8.292 ± 0.74 (18) (b) A
3rd	5.705 ± 0.57 (22) (a)B	6.722 ± 0.66 (18) (a) AB
4th	4.679 ± 0.56 (14) (a) BC	7.833 ± 0.44 (16) (b) AB
5th	4.563 ± 0.66 (14) (a) BC	6.625 ± 0.35 (16) (b) AB
6th	3.33 ± 0.35 (11) (a) C	6.1 ± 0.7 (15) (b) AB
7th	-	5.318 ± 0.57 (15) B

* Means within columns followed by the same upper-case letter or within rows followed by the same lower-case letter are not significantly different at the %5 level by Duncan's multiple range test.*Series A*: mature female held with fruit for determination of multiple mating in females *Series C*: mature males without fruit, for determination of multiple mating in males.

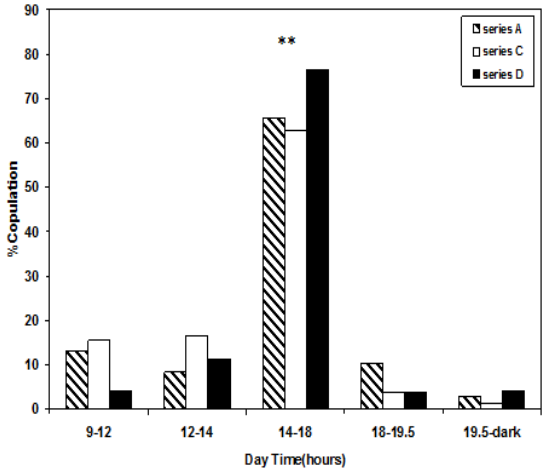

Figure 1. Daily rhythm of mating in *Myiopardalis pardalina* in different series.
Series A: mature female held with fruit for determination of multiple mating in females
Series C: mature males without fruit, for determination of multiple mating in males
Series D: same pair for determination of repeated mating
**: This time period was significantly different with others at the 1% level.

4. Discussion

During the course of the present study, experiments were conducted to investigate the female and male remating with respect to host fruit availability. These data indicate that the Baluchistan Melon fly typically mate more than once regardless of access to host fruit. This finding appears to be opposite to what is known for other species of frugivorous Tephritidae. In Caribbean fruit fly, *A. suspensa*, 60% of females can remate only when they have access to fruit as oviposition site (Sivinski and Heath, 1988). Sixty percent of Mediterranean fruit fly

females held in cages with fruit for oviposition remated (Nakagawa *et al*., 1971) and 50% of the Mexican fruit fly, *Anastrepha ludens* (Loew) females provided wax oviposition domes remated (Robacker *et al.*, 1985). In *T. curvicauda*, remating was observed only when females were kept in cages with both males and immature papaya fruit (Landolt, 1994). Multiple mating in tephritid fruit flies is thought to be due to: (1) forced matings by males controlling access to oviposition sites as in *R. pomonella* (Walsh) (Prokopy and Roitberg, 1984). (2) Poor sperm transfer in initial matings as suspected in part for *C. capitata* (Wiedemann) (Nakagawa *et al.,* 1971), or (3) sperm depletion following extensive oviposition as in *A. suspensa* (Sivinski and Heath, 1988). As Baluchistan melon fly females showed multiple and repeated mating (Hunter *et al.,* 1993) with or without access to host fruit, it is unlikely that multiple and repeated matings are the result of a forced copulation by males or sperm depletion following oviposition. The observed increased remating rates of females held with or without host fruit indicate that poor sperm transfer during matings may be contributed to multiple and repeated matings. An additional possibility is that females need nutrients that may be transferred by males in their ejaculates. In another study, radioactively labeled substances in the ejaculate of *A. suspense* were later recovered in the unfertilized eggs and tissues to mated females (Sivinski and Smittle, 1987). Male Baluchistan melon flies mated like females ($P<0.05$) which is an indication of their potential for polygamy. These results are similar to those reported for males of *C. capitata* (Nakagawa *et al.*, 1971) and *T. curvicauda* that males mated three times more often than females (Landolt, 1994). Copulation duration in series A was shorter than that in series C except for the first copulation ($P<0.03$) (Table 3). It may be hypothesized that the first mating is longer in order to transfer sufficient number of gametes to fertilize all of the female eggs. The other matings may occur to compensate decreased sperm or to take a large number of accessory substances that are transferred with the ejaculate, and which may have a profound effect on female reproductive behavior (Chen, 1984; Gillott, 1988; Eberhard and Cordero, 1995; Eberhard, 1996; Klowden, 1999; Arnqvist and Nilsson, 2000). Also, the long mating duration may be a strategy that male selected for post insemination associations.

In some species, males maintain genital contact for beyond the time needed strictly for insemination of the female (Alcock, 1994). Prolonged copulation has been reported for insects in many orders, including the *Odonata, Phasmida, Lepidoptera, Diptera, Coleoptera, Hymenoptera* and *Heteroptera* (Alcock, 1994). In fruit flies mean mating times vary from 110 second in *Anastrepha pseudoparallela* to 24 hours in *Euarestoides acutangulus* (Headrick and Goeden, 1994; Sivinski *et al*., 2000). The extension of copulation beyond what is required for sperm transfer in related species of fruit flies are often interpreted in terms of sperm competition avoidance (Parker, 1970), protection from predators (Sivinski, 1981), or cryptic female choice (Eberhard, 1996; Belford and Jenkins, 1998; Sivinski *et al*., 2000). Furthermore if the mating continues into the night, beyond the sexual signaling period, when no other males would be searching for mates. However, in some species (e.g., *A. suspensa*), females appear to have a considerable control over mating durations; because males have a difficult time maintaining their position when females become restless and move about (Sivinski et al., 2000). In *M. pardalina*, most of the copulation was observed in the 14-18 hours period in the afternoon (Figure 1). The first mating lasted at least five hours and may be continued into the night. But the following matings lasted shorter than the first (Table 3). So the mating duration can be due to three reasons: (1) compensation of sperm depletion, (2) to take a large number of accessory substances that are transferred with the male ejaculate and (3) post insemination association with maintained genital contact until the dark. The results clearly indicated that male sterility techniques (MST) may not be a successful way to control *M. pardalina*, because females mated more than 5 times during one week. But it seems that the sex attractants could be attractive several times to females and may be useful in their IPM.

Acknowledgments

We gratefully acknowledge the support of the Institute of Plant Pests and Diseases. Also kindly thanks to Prof. Martin Aluja for sending three chapters of his book, Fruit Flies, before publication.Also thanks to anonymus refrees for their good comments.

References

Alcock J. 1994. Post insemination associations between males and females in insects: the mate-guarding hypothesis. A*nnu Rev Entomol*., **39**: 1-21.

Andersson M. 1994. **Sexual Selection**. Princeton, Princeton University Press.

Andrade MCB and Mason AC. 2000. Male condition, female choice, and extreme variation in repeated mating in a scaly cricket *Ornebius aperta* (Orthoptera: Gryllidae: Mogoplistinae). *J Insect Behav.*, **13**: 483-497.

Arnqvist G. 1989. Multiple mating in a water strider: Mutual benefits or intersexual Conflict ? *Anim. Behav*., **38**:749-756.

Arnqvist G and Nilsson T. 2000. The evolution of polyandry: multiple mating and female fitness in insects.*Anim Behav*., **60(2**): 145–164.

Belford SR and Jenkins MD. 1998. Establishing cryptic female choicein nimals.*Trends Ecol. Evol.* **13**: 216-218.

Birkhead TR and Mollar AP. 1992. **Sperm Competition in Birds**. Academic Press, New York.

Brooker MG, Rowley I, Adams M and Baverstock PR. 1990. Promiscuity: An inbreeding avoidance mechanism in a socially monogamous species?.*Behav Ecol. Sociobiol*., **26**:191-199.

Chen PS. 1984. The functional morphology and biochemistry of insect male accessory glands and their secretions. *Annu Rev Entomol*. , **29**: 233-255.

Choe JC. 1995. Courtship feeding and repeated mating in *Zorotypus baberi* (Insecta: Zoraptera). *Anim. Behav*., **49**: 1511-1520.

Eberhard WG. 1996. **Female Control: Sexual Selection by Cryptic Female Choice**.Princeton, New Jersey, Princeton University Press.

Eberhard WG and Cordero C. 1995. Sexual selection by cryptic female choice on male seminal products: a new bridge between sexual selection and reproductive physiology. *Trends Ecol. Evol.*, **10**: 493-496.

Eppo, 2013. *Myiopardalis pardalina* (Dip. Tephritidae) Baluchestan melon fly.available at:www.eppo.int/quarantine/alert_lit/insects/Myiopardalis_pardalina.htm

Fox CW. 1993. The influence of maternal age and mating frequency on egg size and offspring performance in *Callosobruchus maculatus* (Col. Bruchidae). *Oecologia(Berl.)*, **96**: 139-146.

Fox CW, Hickman DL, Raleigh EL and Mousseau TA. 1995. Paternal investment in a seed beetle (Col.Bruchidae): the influence of male size, age, and mating history. *Ann.Entomol.Soc.Am*., **88**: 100-103.

Fuerst PA, Pendlebury WW and Kidwell JF. 1973. Propensity for multiple mating in *D. melanogaster*. *Evolution* **27**: 265-268.

Gillott C 1988. Arthropoda: Insecta, In: Adiyodi,K.G. and Adiyodi,R.(Eds.), **Reproductive Biology of Invertebrates**. Vol. III. Accessory Glands. New York, John Wiley, pp.319-471.

Gomez KA and Gomez AA. 1984. **Statistical Procedures for Agricultural Research.** New York, John Wiley, pp.7-241.

Gwynne DT. 1997. The evolution of edible" sperm sacs" and other forms of courthship feeding in crickets katydids, and their kin (Orth. Ensifera), In: Choe, JC and Crespi, BJ(Eds.), **The Evolution of Mating Systems in Insects and Arachnids**, Cambridge University Press, Cambridge, pp.110-129.

Halliday T and Arnolds SJ. 1987. Multiple mating by females: A perspective from quantitative genetics. *Anim. Behav*., **35**: 939-941.

He Y, Tanaka T and Miyata T. 1995. Eupyrene and apyrene sperm and their numerical fluctuations inside the female reproductive tract of the army worm *Pseudolatia separata*. *J. Insect Physiol*., **41**: 689-694.

Headrick DH and Goeden RD. 1994. Reproductive behavior of California fruit flies and the classification and evolution of Tephritidae(Diptera) mating systems.*Stud.Dipterol*. **1**: 194-252.

Hunter FM, Petrie M, Otronen M, Birkhead T and Mollar AP. 1993. Why do females copulated repeatedly with one male? *Trends Ecol.Evol*. **8**: 21-26.

Jennions MD. 1997. Female promiscuity and genetic incompatibility.*Trends Ecol. Evol*., **12**: 251-253.

Jennions MD and Petrie M. 2000. Why do females mate multiply? A review of the genetic benefits. *Biol. Rev. Camb. Philos. Soc*., **75(1)**: 21-64.

Jimenez-Perez A and Wang Q. 2003.Effect of mating delay on the reproductive performance of Cnephasia jactatana (Lepidoptera : Tortricidae). *J.Econ.Entomol*., **96(3)**: 592-598.

Johnson K and Burley NT. 1997. Mating tactics and mating systems of birds. *Ornithological Monographs*, **49**: 21-60.

Klowden MJ. 1999. The heck is in the male: male mosquitoes affect female physiology and behavior. *J Am Mosq Cont Assoc*., **15**: 213-220.

Koga T, Backwell PRY, Jennions MD and Christy JH. 1998. Elevated predation risk change mating behavior and courtship in a fiddler crab. *Proc R Soc Lond B*, **265**: 1385-1390.

Landolt PJ. 1994. Mating frequency of the Papaya fruit fly (Dip. Tephritidae) with and without host fruit. *Florida Entomol*., **77(3):** 305-312.

Landolt PJ. 2000. Topics in the evolution of sexual behavior in the Tephritidae, In: Aluja, M. and Norrbom, A.L.(Eds.), **Fruit Flies(Tephritidae): Phylogeny and Evolution of Behavior**, Roca Raton, London, New York, Washington, CRC Press, PP. 751-792.

Lens L, Van Dongen S, Van Den Broeck M, Van Broeckhoven C and Dhondt AA. 1997. Why female crested tits copulate repeatedly with the same partner: Evidense for the mate assessment hypothesis. *Behav. Ecol.*, **8**: 87-91.

Madsen T, Shine R, Loman J and Hakansson T. 1992. Why do female adders copulate so frequently? *Nature*, **355**: 440-441.

Nakagawa S, Farias GJ, Suda D, Cunningham RT and Chambers DL. 1971. Reproduction in the Mediterranean fruit fly: frequency of mating in the laboratory. *Ann. Entomol. Soc. Am.*, **64**:, 949-950.

Parker GA. 1970. Sperm competition and its evolutionary consequences in the insects. *Cambridge Phil. Soc. Biol. Rev*., **45**: 525-567.

Petrie M. 1992. Copulation frequency in birds: why do females copulate more than once with the same male? *Anim. Behav.*, 44: 790-792.

Petrie M, Hall M , Halliday T, Budgey H and Pierpoint C. 1992.Multiple mating in a lekking bird: Why do peahens mate with more than one male and with the same male more than once?. *Behav. Ecol. Sociobiol*., **31(5)**: 349-358.

Prokopy RJ and Roitberg BD. 1984. Foraging behavior of true fruit flies. *Am. Sci*., **72**: 41-49.

Reynolds JD. 1996. Animal breeding systems. *Trends Ecol. Evol*. **11**: 68-72.

Ridley M. 1988. Mating frequency and fecundity in insects. *Biol. Rev.*, **63**: 509-549.

Ridley M. 1993. Clutch size and mating frequency in parasitic Hymenoptera. *Am Nat*., **142**: 893-910.

Robacker DC, Ingle SJ and Hart WG. 1985. Mating frequency and response to male-produced pheromone by virgin and mated females of the Mexican fruit fly. *Southwest. Entomol*., **10**: 215-221.

Rubenstein DI.1984. Resource acquisition and alternative mating strategies in water striders. *Am Zool.*, **24**: 345-353.

SAS Institute, 1997. SAS/STAT User's Guide for Personal Computers.SAS Institute, Cary, NC.

Sirjanii M. 1995. Survey on fruit flies active on Cucurbitaceae plants and biology of the important species in Kashmar region of Iran. M.Sc. Thesis, Chamran University, P. 94.

Sivinski J. 1981. The effects of mating on predation in the stick insect *Diapheromera veliei* . *Ann. Entomol. Soc. Am.*, **73**: 553-556.

Sivinski J. and Smittle B. 1987. Male transfer of materials to mates in the Caribbean fruit fly, *Anastrepha suspensa* (Dip.Tephritidae). *Florida Entomol*., **70**: 233-238.

Sivinski J and Heath RR. 1988. Effect of oviposition on remating, response to pheromones and longevity in the female Caribbean fruit fly, *Anastrepha suspensa* (Dip.Tephritidae). *Ann. Entomol. Soc. Am.*, **81**:1021-1024.

Sivinski J, AlujaM , Dodson G, Freidberg A, Headrick D, Kaneshiro K , Thornhill R and Alcock J. 1983. **The Evolution of Insect Mating Systems**. Cambridge, Massachusetts, Harvard University Press.

Zeh JA and Zeh DW. 1996. The evolution of polyandry I : interagenomic conflict and genetic incompatibility. *Proc. R. Soc. Lond. B*, **263**: 1711-1717.

JJBS

Volume 7, Number 3, September .2014
ISSN 1995-6673
Pages 217 - 225

Jordan Journal of Biological Sciences

Relationship of Biometric Size-Weight, Nutritive Value, and Metal Concentrations in *Clarias lazera* (Cuvier and Valenciennes) Reared in Treated Wastewater

Manal M. A. Awad Elkareem[1], Abeer M. H. Karrar[2] and Abdel Karim S. Ali[3,*]

[1]Department of Biology and Biotechnologies, Faculty of Science and Technology, Al Neelain University; [2]Environment and Natural Resources Research Institute, National Center for Research, Ministry of Science and Communication, [3]Department of Environmental Sciences, Faculty of Science and Technology, Al Neelain University, Khartoum, P.O. Box 12702, Sudan

Received: May 17, 2014 Revised: July 3, 2014 Accepted: July 9, 2014

Abstract

The objective of the present study was to investigate the nutritive value and heavy metals accumulation in the flesh of *Clarias lazera* (Cuvier and Valenciennes) reared in the discharge canal of Soba wastewater treatment station, south Khartoum, Sudan. A total of 57 fish were collected from the canal and 52 from the White Nile which served as the control. The proximate composition of fish and concentrations of eight hazardous heavy metals i.e. chromium (Cr), iron (Fe), copper (Cu), zinc (Zn), lead (Pb), robidium (Rb), strontium (Sr), and mercury (Hg) in the flesh of *C. lazera* were measured. The results which were statistically analyzed revealed insignificant differences in moisture, ash, fat contents, energy value, and fat: protein ratio ($P>0.05$) between the two studied sites. A significant difference ($P<0.05$) was evident in the protein content of fishes from both locations. Heavy metals accumulation in *Clarias* tissues differ from one element to another depending on each element characteristics and local environmental conditions e.g. Sr was higher in the White Nile fishes than in treated wastewater fishes ($P<0.05$). Accumulation of Pb and Hg was comparable in wastewater and White Nile ($P>0.05$). Fe, Cu, Zn and Rb concentrations were significantly higher in treated wastewater fishes than natural water fishes ($P<0.05$). Cr was also higher in treated wastewater fishes ($P>0.05$). Nevertheless, the concentrations of most considered elements were lower than levels recommended by various international agencies.

Key words:*Clarias lazera*, Heavy metals, Accumulation, White Nile, Treated wastewater.

1. Introduction

Treated wastewater was used in 19th century in Europe to irrigate crops (Ensink and van der Hoek, 2007). Reuse of treated wastewater for fish aquaculture is practiced in many countries including India (Bunting, 2006), Egypt (Misheloff, 2010), and Netherland (Oberdieck and Verreth 2009). As water demand becomes an increasingly important concern in many places, and especially essential for the increasing human population, fish farming falls within the many options which exist for productive wastewater treatment design systems (WHO, 2006). Popular fish species suitable for fish farming in treated wastewater include catfish, tilapia spp. trout, carp, and many others. The annual production depends on fish species, local and environmental conditions. According to Girard (2011), there are many constraints to reusing treated wastewater for rearing fish, such as lack of knowledge, limited available sites; rapid urbanization, rapid eutrophication, improved sanitation, rapid industrialization contamination, social and cultural acceptance and climate. The pathogen transmission risk through treated wastewater fish farming represents a controllable risk; i.e., pathogen loads can be reduced to acceptable levels if adequate measures are adopted (Straus 1996). Microbial requirements for waste-fed aqua cultural schemes should be compatible with background levels in natural waters, since the harvesting of fish and other aquatic animals is generally unrestricted and socially accepted.

Fish is one of the most important available sources of animal protein in the tropics, and has been widely accepted as a good source of protein and other elements for the maintenance of a healthy body (Tidewell and Allan, 2001). Heavy metals are persistent contaminants in the environment that come to the forefront of dangerous substances such as cadmium, lead, mercury, copper and zinc that cause serious health hazards to humans and animals (Ahmed *et al.*, 1998). The agricultural and

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

industrial wastes, partially treated or untreated regularly, are being discharged into surface water (Forstner and Wittmann, 2007). Heavy metals present in such polluted waters are absorbed through gills, skin and digestive tract of fish by bio-concentration and bio-magnification. Heavy metals are natural trace components of the aquatic environment, but their levels are increased due to domestic, industrial, mining and agricultural activities (Mance, 1987; Kalay and Canli 2000). At low levels, some heavy metals such as copper, cobalt, zinc, iron and manganese are essential for enzymatic activity and many biological processes. However, the essential metals may also become toxic at high concentrations (Bryan 1976 and Authman *et al.,* 2012). Other metals, such as Cd, Hg, Pb play unknown essential roles in living organisms, and are toxic at even low concentrations (Authman *et al.,* 2013).

Clarias lazera (Cuvier. and Valenciennes) is a freshwater fish which attracts attention as a potential fish for aquaculture (Babiker, 1984). According to Chervinski (1984), presence of a breathing apparatus enables the fish to withstand low level of oxygen and a wide range of temperatures. It is an omnivorous feeder found mainly in shallow waters; young ones feed on ostracods and aquatic insects while adults feed on any potential food like zooplankton and molluscs, but mainly on fish e.g. *Oreochromis niloticus* (Amirthalingam and Khalifa, 1965).

The aim of this study was to highlight the implications of the reuse of treated wastewater for fish culture, and to examine and compare the levels of toxic heavy metals accumulation in the muscle tissue of *C. lazera* from the polluted discharge canal of Soba wastewater treatment station and non-polluted of Jebel Aulia Reservoir on the White Nile, south Khartoum, Sudan. The proximate chemical composition and condition factor of fish were studied as complementary aspects. The present study is expected to shed light on the importance of the reuse of treated wastewater for fish farming and its promising role in contributing to provide a safe food for the increasing Sudanese population.

2. Materials and Methods

A total of 57 specimens of *C. lazera* (Arabic Garmouth) were collected from the effluent (discharge) canal carrying the treated wastewater from the maturation ponds, in addition to 52 specimens from the White Nile in the vicinity of Jebel Aulia Dam (control). Fish samples were collected from local fishermen during the period April 2012-April 2013. The canal (Figure 1) is located in the Southern part of Khartoum State ca. 15 Km, (15°29' 55"; 15°30' 25" N; 32°32' 36" – 32°36' 06" E).

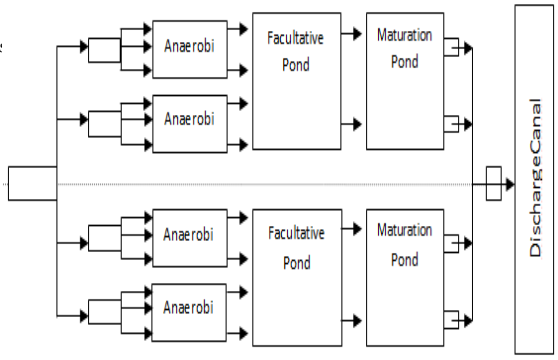

Figure 1: Ponds arrangement, flow pattern and the effluent discharge canal in Soba Wastewater Stabilization Station (Source: Ali and Hag Ibrahim, 2005).

The 2nd site, Jebel Aulia dam on the White Nile, was built in 1937 ca. 50 Km south of Khartoum to store $3.5\mathbf{X}10m^9$ of water (Rzoska *et al.* 1955). The dam length is 5 km and creates a large lake with a width ranging between 1-4 km and a maximum depth 22.5 m. As a result of the dam, a large shallow lake of about 12000 hectares was formed capable of storing 3.5 milliard m^3. The maximum depth of the reservoir is ca. 15 m attained during high flood (August-mid September).

For each fish specimen, the total and standard lengths were measured, and then each fish was weighed. The condition factor (K) was calculated according to Le-Cren, 1951 equation:

$$\mathbf{K= 100.W/L^3}$$

W is fish body weight (g) and L is fish total length (cm)

Fishes were sexed and aged using vertebrae. Use of vertebrae for age determination was proved to be one of the reliable structures for fish ageing (Bishai and Abu Gideiri 1965, Mishrigi 1967, Bishai 1970, Gumaa 1974 and Tweddle 1975). Otolith was not used for aging *C. lazera* because its major advantage over other tissues e. g. scales and vertebrae is the presence of clearly visible daily lines (Pannella 1974). In addition, preparation of otoliths for light microscopy or scanning microscope (Liew 1974) is rather difficult and requires facilities not available in our institutes. Sexual maturity was determined according to Nikoliskii (1963). The stomach food content was investigated using the method described by Hynes (1950) where stomachs contents are examined and the individual food organisms was sorted and identified. The number of stomachs in which each item occurs is recorded and expressed as a percentage of the total number of stomachs examined. The collected fish were then skinned; the flesh was taken from different sites of the body to make sure that the examined sample is well representative to the whole body. Flesh samples were kept in air-tight plastic bags and frozen at $0.0\text{-}5.0^0$C till they were used for moisture and ash contents determination.

The frozen samples were then freeze-dried for 24 hrs using (Edwards High Vacuum 2507 Freeze Dryer). The dried samples were ground using a non metallic mortar. Powder samples were then kept in the air-tight plastic bags. All chemical constituents were determined according to Pearson 1976 and AOAC 1980. These include moisture; protein, fat and ash contents. Protein was evaluated by Micro-Kjeldahl Method; fat content by using soxhelt extraction method. The energy value was calculated from the fat and protein contents of samples using the values 9.02 Kcal./gm for fat content and 4.27 Kcal./gm for protein content as recommended by FAO (1989).

X-ray fluorescence spectroscopy (XRF) was used for qualitative and quantitative determination of heavy metals (Tertian and Claisse 1982). These are namely: Cr, Fe, Cu, Zn, Pb, Rb, Sr and Hg. According to Talbot (1987), XRF has the potential and capacity to give accurate linear response to a broad spectrum of elements in approximately 0.0-500 mg/Kg-1.

The data obtained was statistically analyzed using SPSS package (t-test for normal data and Mann Whitney u test for data not normally distributed).

3. Results and Discussion

The results of the present study (Table 1) showed greater sizes and weights of fishes in treated wastewater compared to those collected from the White Nile (P<0.05). The same applies to standard lengths although the difference was insignificant (P>0.05). According to Mason 1991, such results are possibly attributed to the availability of food in treated wastewater and the presence of organic matter which is rich in proteins, carbohydrates, and fats.

Table 1: Weights and standard lengths of *C. lazera* in treated wastewater and White Nile

Parameter	Treated Wastewater	White Nile	*P*
Weight (gm)	528.96±38.9	360±45.90	*
Standard length	35.04±1.14	30.94±1.21	NS

The condition factor of fish reared in both study sites was calculated to evaluate fish situation. The mean condition factor of fish was found to be 1.23 g/cm^3 and 1.22 g/cm^3 in treated wastewater and White Nile, respectively. Both values were above 1.0 indicating good conditions of the fish (Barnham and Baxter 1998), however, they were below those obtained by Nwabueze 2013 for *Clarias anguillaris*. Furthermore, King (1995) attributed differences in condition factors of fish to food abundance, adaptation to the environment and gonadal development. According to Aloo (1999), the intensity of infection with *Contracaecum* was increased with the increase in the size of bass *Micropterus salmoides*, from Lake Naivasha and the Oloidien Bay, Kenya

Only two stages "I and IV" were observed out of Nikoliskii (1963) six stages for sexual maturity (Stage 1, Immature; Stage 11, quiescent; Stage 111, maturing: Stage 1V, mature: Stage V running; Stage V1, Spent).

Table 2 illustrates the difference in body weight according to sex, age and maturity stage and the results gave an insignificant difference with sex and maturity stage (P>0.5) in both localities. However, weights of mature fish were higher than immature fish. Regarding age, a significant difference was encountered (P<0.05) in weights of fish; older fish weights were higher than younger ones in both sites.

Figure 2 shows the moisture, ash, fat and protein contents which were examined to assess the fish flesh quality. The fat/protein ratio was calculated to indicate the nutritive value of flesh. Protein content in wastewater fish was higher than in the White Nile (P<0.05). Fat, moisture content and fat/protein ratio were almost similar (P>0.05) in the two sites (0.212 for Treated wastewater and 0.217 for the White Nile).

The food value of fish is normally estimated as the percentage of the edible portion to the total weight of fish and its contents of the basic nutrients i.e. fats and proteins (Karrar, 1997). In the present study, the energy values calculated were 0.0683 and 0.0629 for wastewater and White Nile fish, respectively. These figures indicate that the nutritive values of the fish from both study sites are comparable; however, freshwater fishes are slightly more nutritious due to their low fat/protein ratio.

Table 2: Variation in body weights of *C. lazera* in treated wastewater and White Nile according to sex, age group and maturity stage

	Sex		Age Groups				Maturity stage			
	Treated wastewater	White Nile	Treated wastewater		White Nile		Treated Wastewater		White Nile	
Male	459.38± 63.5	392.00±86.60	1	427.20±29.8	1	308.60±29.30	A	371.04±33.5	336.8±58.7	
Female	575.35+63.50	333.28±42.20	2	936.03±53.1	2	1480.00±10.0	B	714.40±53.7	426.4±10.0	
P	NS	NS	*		*		*		*	

*significant; NS insignificant

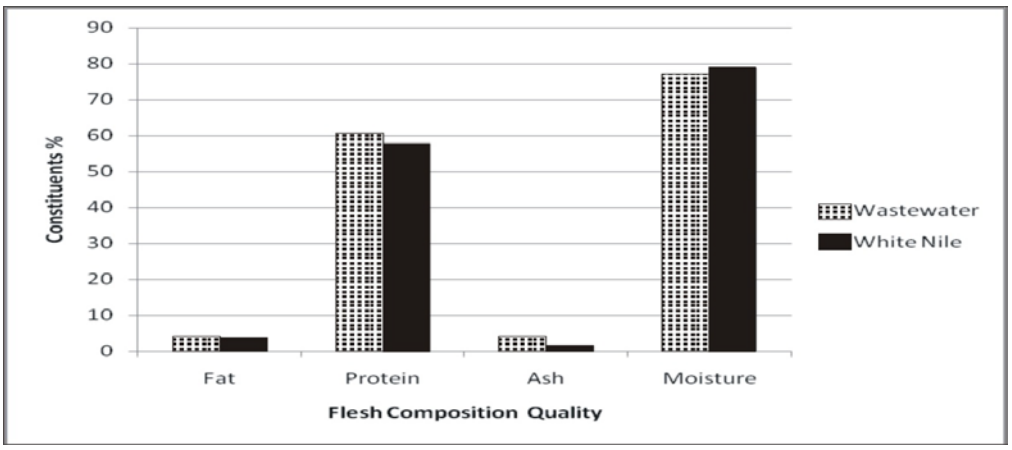

Figure2. Chemical composition of *C. lazera* in treated wastewater and White Nile

The energy value was higher in the White Nile fish (445.911+17.23 Kcal / 100 gm) than in wastewater (414.540±8.394 Kcal / 100 gm), but the difference was insignificant (P>0.05).

Figures 3a, 3b and 3c illustrate no significant difference in fat, protein and ash contents with sex, age and maturity stage, respectively (P>0.05). Exceptions were encountered in females' fat content in the White Nile that showed significantly higher values than in males (P<0.05).

Proximate composition showed no significant variation according to sex in both localities. This finding is in agreement with Mohammed *et al.* (1988) who assessed the chemical composition of *Mugil cephalus* along the Sudanese Red Sea Coast. Also proximate composition showed no significant variation with age. That result was in contrast to Dambergs (1963), but older fish reflected slight insignificant increase in fat and protein contents.

Protein and ash contents were insignificantly higher in mature fish in both study sites and that was also in contrast to Dambergs (1963), but fat content showed insignificantly higher levels in the immature fish. According to Hoar (1957), fat storage in the muscle tissue increased prior gonads maturation to provide energy for spawning activities.

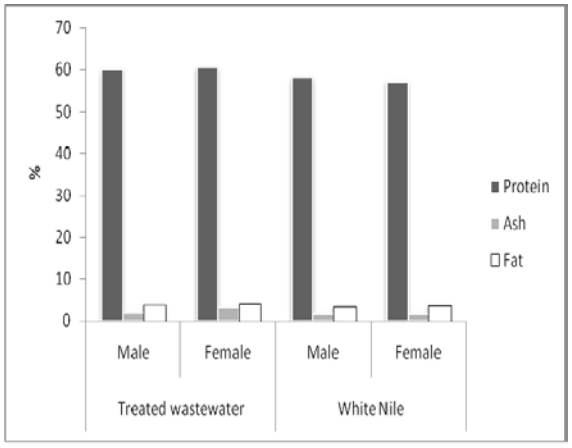

Figure 3a. Variation in Chemical composition of *C. lazera* in wastewater effluent canal and White Nile according to sex

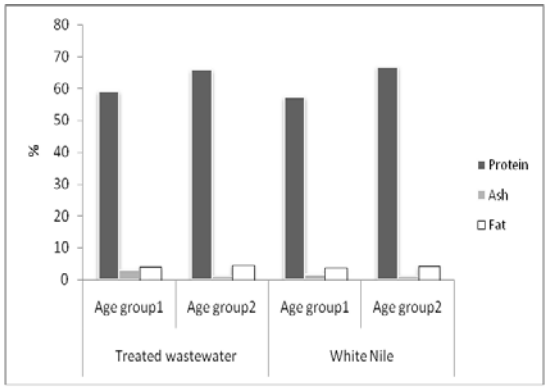

Figure 3b. Variation in Chemical composition of *C. lazera* in wastewater effluent canal and White Nile according to age group (age group 1: 1, 2 and 3 years; Age group 2: 4 and 5 years)

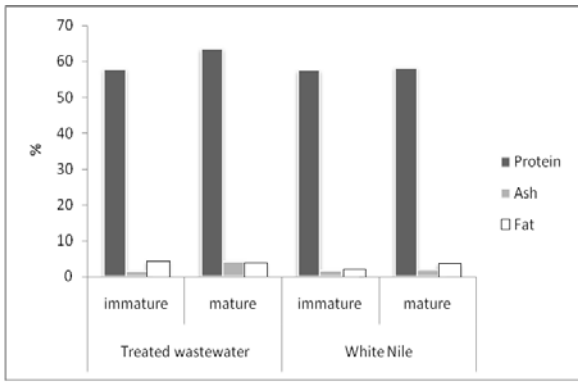

Figure 3c. Variation in Chemical composition of *C. lazera* in wastewater effluent canal and White Nile according to age

Concentrations of Fe, Cu, Zn and Rb (Table 3) were significantly higher in treated wastewater fishes than natural water fishes (P<0.05). In view of this, they were further analyzed with sex, age group, and maturity stage (Figures4a, 4b, 4c). Fe and Zn showed no variation with all the previously mentioned parameters in both study sites (P>0.05). Cr was also higher in treated wastewater fishes (P>0.05). Hg and Pb concentration was higher in treated wastewater than the White Nile. Statistically, the two heavy metal expressed insignificant difference ((P>0.05). Sr was significantly higher in the White Nile compared with treated wastewater (P<0.05).

Table 3. Heavy metals concentrations in *C. lazera* in treated wastewater and White Nile

Conc. (ppm)	Wastewater	White Nile	*P*	Permissible limits (µg/g wet weight)
Cu	4.86± 0.56	1.26± 0.16	*	3.280 "IAEA 2003; FAO 1983a"; 20 "MAAF 2000"
Rb	177.63± 23.18	12.89± 1.54	*	2.86 "IAEA 2003"
Cr	0.05± 0.02	0.03±0.01	NS	0.730 "IAEA 2003"
Fe	72.64± 12.60	6.94±0.93	*	146 "IAEA 2003"
Zn	497.20± 44.7	37.83±7.04	*	30 FAO 1983a; 40 FAO/WHO 1989
Hg	60.03 ± 1.30	57.42±3.32	*	0.222 "IAEA 2003"; 0.50 "EC 2006"
Pb	0.17± 0.02	0.09±0.01	*	0.2 "EC 2005"
Sr	2.7 3 ± 2.46	17.55 ± 10.35	*	130 "IAEA 2003"

These results are in agreement with the findings of many authors e.g. Clement and Lovel (1994) and Gomez (2011). However, ash content was significantly higher in treated wastewater fishes ($P<0.05$). The concentrations of most elements considered in this study were lower than levels recommended by the international agencies e.g.EC (2006); EC (2005); IAEA (2003); FAO/WHO (1989); FAO (1983a). Rb showed significant variation with sex and age in both study sites but not with maturity stage. Cu concentration showed no variation with sex in both study sites ($P>0.05$). However, it gave significant differences with age in the two studied sites. With maturity stage, Cu was much higher in treated wastewater fish and expressing a significant difference ($P<0.05$).

Treated wastewater fish were found to accumulate considerable amounts of heavy metals compared with White Nile fish. This is likely because metals have certain properties which make them difficult to treat or remove from wastewater if not treated by certain chemical methods (Mubarak, 2014), and living organisms usually form an intimate relationship with the chemical composition of their environment (Elgobashy *et al*. 2001). According to Jezierska and Witeska (2006), fish living in polluted waters tend to accumulate heavy metals in their tissues. Generally, accumulation depends on metal concentration, time of exposure, way of metal uptake, environmental conditions (water temperature, pH, hardness, salinity), and intrinsic factors (fish age, feeding habits). Concentrations of Pb and Hg in treated wastewater fish were almost similar to fish of natural non-polluted water possibly because surface water does not always reflect the real situation of heavy metals pollution. This is expected since the physico-chemical characteristics of water normally affect the distribution of metals in water. In addition, Karrar (1997) reported that some metals have the property of being rapidly adsorbed to particulate materials like detritus and suspended sediments. Moreover, some biological processes like uptake by planktons through assimilation can exert the same effect (Abu Gideiri, 1980).

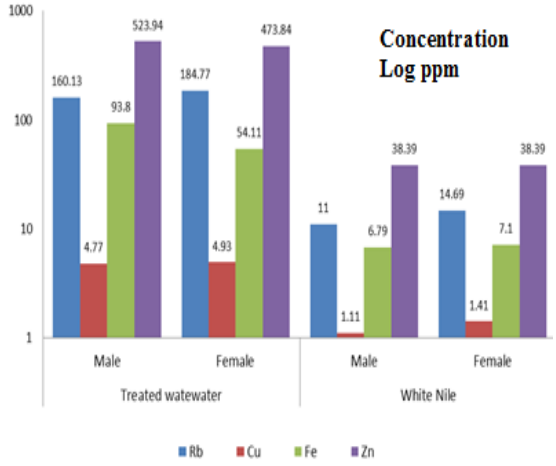

Figure 4a. Heavy metals accumulation in *C. lazera* in wastewater effluent canal and White Nile according to sex

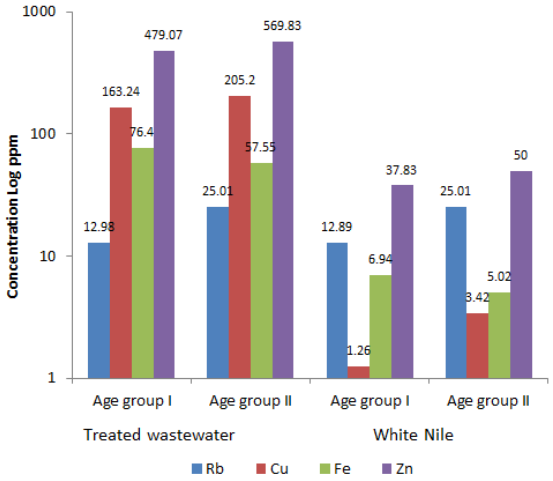

Figure 4b. Heavy metals accumulation in *C. lazera* in wastewater effluent canal and White Nile according to age

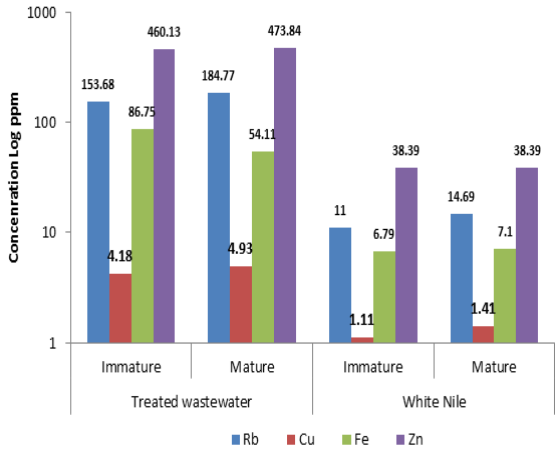

Figure 4c. Heavy metals accumulation in *C. lazera* in wastewater effluent canal and White Nile according to maturity stage

Cu, Rb, Fe and Zn showed significantly higher levels of accumulation in fish of treated wastewater than White Nile fish ($P<0.05$) probably because their concentration was lower in the latter site. However, except for Zn, the concentrations of other metals did not exceed the permitted international levels (FAO 1983a, b; Huss, 1994; Al-Wher, 2008). Cr, Hg and Pb reached the wastewater

treatment plant with the wastewater transported from the Khartoum Industrial Area e.g. Khartoum Tannery, Printing facilities, paints factories and other industrial activities (Sabir *et al.* 2007). These metals were not subject to any biodegradation since the station depends mainly on natural micro-organisms which can only decompose organic matter. Zn, Fe and Cu concentrations were higher than Hg, Cr and Pb in both sites. Such situation could be attributed to the fact that Zn, Fe and Cu are essential micro-nutrients required in life processes, so most organisms have the ability to keep them at high concentrations in their bodies. This capacity is enhanced by certain feeding and metabolic processes which lead to high accumulation. Furthermore, many of these metals are capable of forming complexes with the available organic substances and hence have the tendency to be fixed in tissues rather than being excreted (Mara and Cairncross 1989). Deposition of heavy metals in fish tissues in treated wastewater seems to inflict no harmful effects on *Clarias* which survive successfully with increasing numbers and sizes. In conformity with this, Zaki (2007) stated that aquatic fauna are exposed to chronic substances i.e. pollutants that do not cause heavy mortalities but fishes survive and accumulate various amounts of microbial or chemical residues of heavy metals which might result, in extreme cases, in unpleasant tastes or are potentially dangerous. Saeed (2007) results on *C. gariepinus* in Lake Edku, Egypt, revealed that accumulation of heavy metals generally associated with specific tissues/organs of the fish e.g. Cu in liver, Cd in gills, liver and ovary and kidney and Pb in gills and ovary. Furthermore, Benamar and Zitouni (2013) reported that accumulation of Cu is higher than Cr in liver tissue compared with the muscle tissue of *Sardinella aurita* collected from Oran Coastline in Algeria. Das and Gupta (2013) concluded that Cu accumulation pattern in the Indian flying barb *Esomus danricus* was related to metal concentration and increased with exposure time. The findings of this study are also in agreement with Mason (1991) and Ebrahimi *et al.* (2007) who reported that consumption of edible tissue of mullet which is subjected to heavy metals pollution is not harmful to humans and that the heavy metals accumulation in fish tissues is below the Egyptian standards. Presence of some organic substances in treated wastewater may render heavy metals less toxic than they would be in pure uncontaminated conditions. The form of heavy metal to which aquatic organisms are exposed is important and determines its overall toxicity (Singh *et al.* 2011).

Cu, Rb, Fe and Zn showed slightly different pattern when analyzed according to sex. Values of Fe, Cu, and Zn showed insignificant variation between males and females in both study sites, while Rb showed significant increase in female specimens from both study areas which may be due to the high ash content of females. Patin (1982) however, reported a positive correlation between the ash content of the fish and its content of a certain metal. Authman and Abbass (2007) findings on heavy metals in *Tilapia zilli* and *Mugil cephalus* tissues indicated that accumulation was much higher than concentration of these heavy metals in their surrounding waters. The water characteristics for the two sites, obtained by Sabir *et al.* (2007) were in conformity with the results of the present study.

According to age, Cu, and Rb showed significantly higher accumulation in older fish in both localities, Zn insignificantly showed the same (Figure 4b). That is likely because fish accumulate elements throughout their life, so older fish would contain more of these substances (Connel 1975; Huss 1994). The previous finding is in agreement with Patin (1982) who found positive correlation between the actual metal content of a fish and its age, size and weight. Fe although showed no variation with age, the difference was not significant.

The pattern of variation of Cu, Rb, Fe and Zn with the stages of maturity of fish from both study sites did not follow certain models although Cu, and Rb showed insignificantly higher levels in mature fish in both study sites and Zn showed the same in treated waste water fish. According to Huss (1994), fish becomes mature when it reaches certain size not a certain age so mature fish with larger sizes are expected to contain more heavy metals as the actual metal content of a fish is positively related to its size and weight.

Insects, phytoplankton, zooplankton, weeds, molluscs and fish parts were detected as major food constituents in the stomach of *Clarias lazera* from both studied localities (Figure 5). Phytoplankton, fish parts and molluscs showed significant variations between the two sites whereas mean values of insects, zooplankton and weeds were almost similar (P>0.05). Fish, molluscs and zooplankton were much more detected in stomachs of freshwater fish because wastewater doesn't suit their growth. According to Jumaa (1974) the partially low oxygen, elevated CO_2 and the relatively high concentrations of nitrogen and nitrates create unfavorable conditions for zooplanktons growth. Besides, metals in both cationic and soluble complex forms can be toxic or inhibit zooplanktons growth (Biesinger and Christenser 1972). Superiority of phytoplankton and insects can be explained as due to presence of abundant organic matter in the treated wastewater which furnishes suitable conditions for their growth.

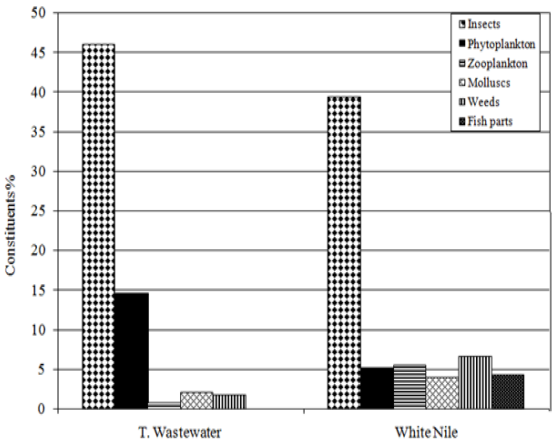

Figure 5. Food composition of *C. lazera* in treated wastewater effluent canal and White Nile

4. Conclusions

Insignificant variations were evident in proximate chemical composition of fish with age, sex, and maturity stages in both sites. However, fish collected from the treated effluent canal expressed higher sizes; weights and protein content are preferred by local consumers. Nutritive value of fishes reared in treated wastewater effluent was high and almost similar to that of natural habitat fish with significantly higher protein content. In spite of possible cultural bias against fish reared in treated wastewater effluents, the average concentrations of most hazardous heavy metals e.g. Hg and Pb are comparable to those obtained from fishes of the White Nile and are well below the values recommended by IAEA (2003), EC (2006) and other relevant agencies for human consumption. Zn concentrations exceeded the recommended level set by international agencies for human consumption and may constitute a potential health risk if ingested in large quantities.

Acknowledgements

Thanks are extended to the staff of the wastewater treatment station, the Atomic Energy Agency, Applied Nuclear Physics laboratory (Department of Physics, University of Khartoum) and Animal Production Section (National centre for Research, Khartoum).

References

Abu Gideiri Y. B. 1980. Metal contamination in central Red Sea. Report to Red Sea Commission.

Ahmed YF, Mohamed MM, El-Nemer IZ, El-Desoky KI and Ibrahim SS. 1998. Some pathological studies on the effect of cadmium and mercuric chlorides on the gonads of catfish (*Clarias lazera*). *Egypt J Comp Pathol Clin Pathol*., **11**: 72-81.

Amirthalingam C and Khalifa MY.1965. A Guide to Common Commercial Freshwater Fishes in the Sudan. Government Printing Press, Khartoum, 197 pp.

Ali A K S and Hag Ibrahim S N. 2005. Soba wastewater stabilization pond (WSP) - Sudan: Treatment performance and microbiological quality. Proceedings Sardinia 2005, 10th *International Waste Management and Landfill Symposium.*

Aloo PA.1999. Ecological studies of helminth parasites of the large mouth bass, *Micropterus salmoides*, from Lake Naivasha and the Oloidien Bay, Kenya. Onderstepoort *J Vet Res*., **66**:73-79.

Al-Wher S M. 2008. Levels of heavy metal Cd, Cu, Zn in three fish species collected from Northern Jordan Valley, Jordan. *Jordan J Biol Sci.,* **1(1):** 41-46.

AOAC 1980. **Official Methods for Analysis**, Horwitz, N. (Ed.), 13th Ed. Association of Official Analytical Chemists, Washington D. C. 957 pp.

Authman M M N and Abbas WT. 2007. Accumulation and distribution of copper and zinc in both water and some vital tissues of two species (*Tilapia zilli* and *Mugil cephalus*) of Lake Garoun, Fayoum Province, Egypt. *Pak J Biol Sci.,* **10(13):** 2106-2122.

Authman M M N, Abbas WT and Gaafar AY. 2012. Metals concentrations in Nile tilapia *Oreochromis niloticus* (Linnaeus, 1758) from illegal fish farm in Al-Minufiya Province, Egypt, and their effects on some tissues structures. *Ecotoxicol. Environ. Saf.*, **84**: 163–172.

Authman M M N, Abbas HH and Abbas WT. 2013. Assessment of metal status in drainage canal water and their bioaccumulation in *Oreochromis niloticus* fish in relation to human health. *Env Mon Asses*., **185(1)**: 891-907.

Babiker M M. 1984. Aspects of the biology of the catfish *Clarias lazera* (Cuv. & Val.) related to its economic cultivation. *Hydrobiol.,* **110(1):** 295-304.

Barnham C and Baxter A. 1998. Condition Factor, K, for Salmonid Fish. Fisheries Notes. State of Victoria, Department of Primary Industries 2003:3 pp.

Benamar N and Zitouni B. 2013. Levels of chromium and copper in liver and muscle tissues of the round *Sardinella Sardinella aurita* (Valenciennes) from the Oran Coastline, Algeria. *Jordan J Biol Sci.,* **6 (4):** 252 – 256.

Biesenger K E and Christenser GM. 1972. Effect of varius metals on survival, growth, reproduction and metabolism of *Daphnia mogna*. *Fish Res Bd. Canada*, **29**: 1691-1700.

Bishai H M and Abu Gideiri YB.1965. Studies on the biology of genus Synodontis at Khartoum I: Age and Growth. *Hydrobiol.,* **26**: 85 – 97.

Bishai H M. 1970. Studies on the biology of family Bagridae in the Sudan. A Ph. D Thesis submitted to Fac. Sci. Cairo University. Eygpt.

Bryan G W. 1976. Some effects of heavy metal tolerance in aquatic organisms. In: Lockwood APM (Ed.) **Effects of Pollutants on Aquatic Organisms**. Cambridge University Press. Cambridge, England. pp. 7.

Bunting S W. 2006. Confronting the realities of wastewater aquaculture in peri -urban Kolkata with bioeconomic modeling. *Water Res.,* **41**: 499-505.

Chervinski J. 1984. Salinity tolerance of young catfish *Clarias lazera*. *J Fish Biol.,* **23**: 147-149.

Clement S and Lovel R T. 1994. Comparison of processing yield and nutrient composition of cultured Nile Tilapia *Oreochromis niloticus* and channel cat fish *Ictaluruspunctatus*. *Aquaculture*, **119 (2 – 3):** 299–310.

Connel J J. 1975. **Control of Fish Quality**: Fishing News Ltd. Surrey, England.

Dambergs N. 1963. Extractives of fish muscle. 3. Amount, sectional distribution, and variations of fat, water solubles, protein and moisture in cod ''*Gadus morhual* L.'' fillets. *J Fish Res Bd Can.,* **20(4):** 909-918.

Das S and Gupta A. 2013. Accumulation of copper in different tissues and changes in oxygen consumption rate in Indian flying barb, *Esomus danricus* (Hamilton-Buchanan) exposed to sub-lethal concentrations of copper. *Jordan J Biol Sci*., **6(1):** 21 – 24.

Ebrahimi G, El Attia MA and Waffek M. 2007. Heavy metals and bacteria distribution in different organs of grey mullet (*Mugil cephalus*) cultured in different environmental conditions. *The International Arab African Fish Resources Conference and Exhibition,* Cairo, Egypt.

EC, 2005. European Community. Commission Regulation No 78/2005 pp.: L16/43–L16/45. *Official Journal of the European Union* (20.1.2005).

EC. 2006. European Community. Commission Regulation (EC) No. 1881/2006 of 19 December 2006. Setting maximum levels for certain contaminants in foodstuffs. OJ L 364.

Elgobashy H A, Zaghloul KH and Metwally MAA. 2001. Effect of some water pollutants on the Nile tilapia, *Oreochromis niloticus* collected from the River Nile and some Egyptian lakes. *Egypt J Aqual Biol & Fish*., **5(4):** 251 – 219.

Ensink J H and van der Hoek W. 2007. Editorial: New international guidelines for wasetwater use in agriculture. *Tropical Medicine and International Health*. **12 (5):** 575-577.

FAO, Food and Agricultural organization 1983a Compilation of legal limits for hazardous substances in fish and fishery products. *Fisheries Circular* No. 764. FAO, Rome.

FAO, Food and Agricultural Organization 1983b. Manual of Analyses of metals and organochlorines in fish. *FAO Fisheries Technical Paper*: 212.

FAO Food and Agricultural Organization 1989. Yield and Nutritional Value of the Commercially More Important Fish Species. *FAO Fisheries Technical Paper* No. 309, Rome, Italy, 187 pp.

FAO/WHO 1989. Evaluation of certain food additives and the contaminants mercury, lead and cadmium, *WHO Technical Report, Series* No. 505.

Forstner N and Wittmann GTW 2007. **Metal Pollution in the Aquatic Environment**. Springer- Verlag, Belin.

Girard J J. 2011. Feasibility of Wastewater Reuse for Fish Production in Small Communities in a Developing World Setting. M. SC. Thesis, Univ. South Florida.

Gomez R G. 2011. Integrated fish farming strategies. World Water Day, 2011. FAO Fisheries and Aquaculture Department: http://www.fao.org/fishery/en

Hoar W S. 1957. The gonads and reproduction. In: Brown ME (Ed), **The Physiology of Fishes.** Vol. 1. Academic Press Inc. Publishers, New York.

Huss H H. 1994. Assurance of sea food quality. *FAO Fisheries Tech. Paper* 334, Rome, Italy.

Hynes H B N. 1950 The food of freshwater of stickleback (*Gasterosteus aculentus* and *Pygosteus pungitus*) with a review of methods used in studies of food of fish. *J Anim Ecol*., **19**: 36-57.

IAEA 2003. International Atomic Energy Agency, Trace elements and methylmercury in Fish Tissue.; -407: 4.

Jezierska B and Witeska M. 2006. The metal uptake and accumulation in fish living in polluted waters. In: Tawardoska I, Allen HE, Haggblom MM and Stefaniak S. (Eds), **Soil and Water Pollution Monitoring, Protection and Remediation.** Springer, Netherlands, pp 107-114.

Jumaa S A. 1974. Fish environment relationships. M. Sc. Thesis. Universityof Khartoum.

Kalay M and Canli M. 2000. Elimination of essential (Cu, Zn) and nonessential (Cd, Pb) metals from tissue of a freshwater fish *Tilapia zillii* following an uptake protocol. *Tuk. J Zool*., **24**: 429-436.

Karrar A M H. 1997. Studies on the biochemical composition of fish and current grading. M. Sc. Thesis, University of Khartoum.

King M. 1995. **Fisheries Biology, Assessment and Management**. Fishing News Books, Oxford, UK.

Le-Cren E D. 1951. The length-weight relationship and seasonal cycle in gonad weight and condition in perch *(Perca fluviatilis). J Anim Ecol.* ,**20**: 201-219.

Liew, P. K. L. 1974. Age determination of American eels based on structure of their otoliths. The Proceedings of an International Symposium on the Aging of Fish. (Edit). Bagenal, T. B. Univ. of Reading, England, 19-20 July, 1973.

MAFF, 2000 Ministry of Agriculture, Fisheries and Food. Monitoring and surveillance of non-radioactive contaminants in the aquatic environment and activities regulating the disposal of wastesat sea, 1997. In Aquatic Environment Monitoring Report No. 52. Center for Environment, Fisheries and Aquaculture Science, Lowestoft, UK.

Mance G. 1987. **Pollution Threat of Heavy Metals in Aquatic Environment**. Elsevier, London.

Mara D and Cairncross S. 1989. Guidelines for the safe use of wastewater and excreta in agriculture and aquaculture. Measures for public health protection. World Health Organization, Geneva.

Mason C F. 1991. **Biology of Fresh Water Pollution**. Longman Singapore publishers, Singapore.

Misheloff, R. 2010. Integrated water resource management II feasibility of wastewater reuse. United States Agency for International Development (USAID). Report No. 14. 809 pp.

Mishrigi S Y. 1967. Study of Age and growth in Lates niloticus at Khartoum. *Hydrobiol.*, **30**: 45 – 56.

Mohammed G H, Mahmoud ZN and Elhag EA 1988. Chemical composition of *Mugil cephalus* of the Sudanese Red Sea Coast. *Sudan J Sci*., **3**: 10-17.

Mubarak N M, Sahu JN, Abdulah EC and Jayakumar M. 2014. Removal of heavy metals from wastewater using carbon nanotubes. *Separation and Purification Rev.*, **43(4):** 311-338.

Nikolskii G V. 1963. **The Ecology of Fishes.** Academic Press, London, New York. 352 pp.

Nwabueze A A. 2013. Growth performance of the mudfish, *Clarias anguillaris* (Pellegrin,

1923) in Treated and Untreated Domestic Sewage. *Sustainable Agriculture Res.*, **2 (1):** 62-69.

Oberdieck A and Verreth J. 2009. A handbook for sustainable aquaculture. integrated approach for a sustainable and healthy freshwater aquaculture. Sixth Framework Programme. 111 pp

Pannella G. 1974. Otolith growth patterns: an aid inage determination in temperate and tropical fishes. The Proceedings of an International Symposium on The Aging of Fish. (Edit). Bagenal, T. B. Univ. of Reading, England, 19-20 July, 1973.

Patin S A. 1982. **Pollution and the Biological Resources of the Oceans**. (English Translation). Butterworth and Comp. Ltd. England.

Pearson D. 1976. **The Chemical Analysis of Foods**. 7th edition, Churchill, Livingstone, Edinburgh, London and New York.

Rzoska J, Brook J and Prowse G A. 1955. Seasonal plankton development in the White Nile and the Blue Nile near Khartoum. *Verh. Int. Ver. Limnol*.,**12**: 327-337.

Sabir A A, Ahmed A A and Ali A K S. 2007. Environmental impact on biodiversity of micropopulations in three water bodies in Khartoum State, Sudan. *Egypt J Aquat Biol & Fish*., **11(3):** 527-543.

Saeed S M. 2007. Accumulation of heavy metals in different tissue/organs of the Nile catfish, *Clarias gariepenus* at Lake Edku, as biomarker of environmental pollution**.** The International Arab African Fish Resources Conf. and Exhib. 28-30 June, Cairo, Abstract 36.

Singh R, Gautam N, Mishra A, and Gupta R.2011. Heavy metals and living systems: An overview.*Indian J Pharmacol.*, **43(3):** 246–253

Strauss M. 1996. Health (Pathogen) Considerations Regarding the Use of Human Waste in Aquaculture. Water and Sanitation in Developing Countries.

Talbot V. 1987. Rapid multielement analysis of oyster and cockle using XRF fluorescence and spectroscopy, with application to reconnaissance marine pollution investigations. *Sci Tot Env*., **66**: 213-223.

Tertian R and Claisse F. 1982**. Principles of Quantitative X-ray Fluorescence Analysis.** Hyden and Son Ltd., London, Philadelphia and Kheine.

Tidewell JH and Allan, G. L. 2001. Fish as food: aquaculture's contribution. *EMBO Rep*. **2(11):** 958–963.

Tweddle D. 1975. Age and growth of the catfish, *Bagrus meridionalis* in Southern Lake Malawi. *J Fish Biol.*, **7**: 677 – 685.

WHO 2006. Guidelines for the safe use of wastewater, excreta and greywater. Volume 3 Wastewater and excreta use in aquaculture. World Health Organization, 158 pp.

Zaki M S. 2007. Impact of heavy metals pollution on fishes. *Proceedings of Egyfish (2007) Conference*, Cairo, Egypt.

JJBS

Volume 7, Number 3, September .2014
ISSN 1995-6673
Pages 227 - 231

Jordan Journal of Biological Sciences

Microscopic Analysis of Extruded and Pelleted Barley and Sorghum Grains

Mohammed A. Bdour[1], Ghaid J. Al-Rabadi[1,*] , Nofal S. Al-Ameiri[2], Atif Y. Mahadeen[1] and Muhammad H. Aaludatt[3]

[1]Department of Plant Protection;[2]Department of Protection and Integrated Pest Management, Faculty of Agriculture, Mutah University, Al-Karak 61710;[3] Department of Food Science and Nutrition, Faculty of Agriculture, Jordan University of Science and Technology, Irbid 22110, Jordan.

Received: April 29, 2014 Revised: June 26, 2014 Accepted: July 11, 2014

Abstract

Scanning Electron Microscopy (SEM) technique has been used in food and feed industry to characterize the final product with the main focus on changes that occur to starch material. This study demonstrates the use of SEM for examining the changes that occur to starch granules after exposing sorghum and barley grains to mild (pelleting) and intensive (extrusion) feed processing methods. In this study, SEM images analysis is proved to be a useful tool to recognize the changes that occur to grains after a different processing method. The present study showed that the swelling and melting of starch granules are influenced by the severity of the processing method. In case of pelleting process, oval shape appearance of starch granules remained intact after the pelleting process; however, absence of intact oval shape of starch granules occurred after the extrusion process.

Keywords: Grains, Scanning Electron Microscopy, Gelatinization, Starch, Feed Processing.

1. Introduction

Grains usually represent the main ingredient component in both ruminant and monogastric animals feed and are considered the primary energy source (Svihus *et al*., 2004). Before feeding animals, grains are ground to increase the digestibility and to improve mixing with other feed ingredients (Al-Rabadi *et al*., 2009). Due to the incomplete starch digestion, grains are further processed to enhance starch gelatinization and thus digestibility (Svihus *et al.*, 2005). Excellent positive correlations have been reported between extent of starch gelatinization and digestibility among animal feed (Svihus *et al.*, 2005). Extent of starch gelatinization is dependent on the processing method (pelleting, steam flaking, expanding, extrusion) and operating variables within the processing method (level of water addition, temperature and retention time) (Gilpin *et al*., 2002). The pelleting process is the most conventional method in producing animal feed where feed material is exposed to steam and then forced through a die (Thomas *et al.*, 1997). The extrusion process is defined as a high temperature short time treatment where feed material is exposed to friction and shearing forces. Different microscopy techniques, such as scanning electron microscopy (SEM) have been reported to be applied into food and feed industry for quality control purposes and particularly in cereal products to determine the extent of starch gelatinization and characteristics in final product (Lee *et al*., 2000; Srikaeo *et al.*, 2006; Srikaeo, 2008; Olav and Svihus, 2011). The objective of this study was to use microscopic analysis to examine the influence of two different feed processing methods (steam pelleting and extrusion) on starch structural changes of processed sorghum and barley grains used for animal feed.

.

2. Materials and Methods

2.1. Grain Processing (Steam Pelleting)

Sorghum and barley grains were obtained from the Queensland Department of Primary Industry and Fisheries, Australia. Grains were milled under steady state conditions using 4 mm hammer mill screen size (i.e., when there is no change in motor load or ampere meter reading) before being steam pelleted (Ring Die 520 diameter, Munch Edelstahl, Hilden, Germany) under constant motor load. For both barley and sorghum grains, steam conditioning temperature was 85 ˚C and moisture added as a steam at pre-conditioner at rate of 1.9 and 2.5%. Pellet diameter and length were 4.0 and 6.0 mm,

* Corresponding author. e-mail: bdour2002@yahoo.com.

respectively. After the pellets were prepared, they were sealed into 25 kg plastic bags and stored at 4 ºC before examination using scanning electron microscopy.

2.2. Grain Processing (Extrusion)

High-temperature short-time (HTST) extrusion cooking was conducted using a co-rotating twin-screw model Prism Eurolab KX16 (Thermo Prism, Stafford-shire, UK). The barrel diameter was 16 mm with a length/diameter ratio of 40:1. The die had two openings each 2 mm in diameter and 8 mm in length. Melt pressure was measured with a pressure transducer fitted to the die block (Terwin, Nottinghamshire, UK). Motor torque, screw speed, barrel temperatures and melt pressure were monitored with Prism software (Sysmac-SCS version 2.2; Omron Corporation, Milton Keynes, UK). Die temperature ranged from 90-100 ˚C and die pressure ranged 4.85-16.61 bar. Liquid feed rate and dry feed rate were recorded manually after being calibrated before processing. Dry feed was fed through a single screw volumetric feeder (KX16 Powder feeder; Brabender Technology, Duisburg, Germany). Water was injected through a port 150 mm from the start of the barrel using a peristaltic pump (L/S 7523) with a Tygon Lab tubing 13 (0.8 mm internal diameter, Masterflex; Cole-Parmer Instrument Company, Vernon Hills, IL, USA). The dry feed rate for barley and sorghum was 20 g/min and 25 g/min, respectively, and the amount of water added at the extruder barrel was adjusted to compensate for moisture differences in the samples to have a dough moisture content of 55% for barley and 50% for sorghum (wb). Barley fractions were extruded at lower feed rate and higher moisture content, compared to sorghum, to avoid any possible blockage during extrusion. High barrel temperature settings (140 ˚C) and constant screw speed of 200 r.p.m. were used.

Samples were collected when the extruder was running at a steady state (i.e., stable values for both torque and die pressure). The samples were collected over 15–20 min., placed in an aluminium tray, and dried in a hot air oven (50 ˚C for 24 h) (Ballogou *et al.,* 2011). After drying, they were sealed into plastic bags and stored at -18 ˚C pending visual examination by using scanning electron microscopy.

2.3. Scanning Electron Microscopy (SEM)

Specimens were mounted onto aluminium stubs with carbon tabs and sputter coated with a 10-15 nm layer of platinum using an EIKO IB-5 Platinum Sputter Coater. Samples were viewed by field emission scanning electron microscope (JEOL 6300 or JEOL 6400, Japan). Representative Scanning Electron Micrographs were selected by taking many 5 to 10 pictures for the selected sample. The selected sample contains many grain fragments on the carbon tabs. For each grain fragment, many pictures were taken at different magnifications (range from 25-1500X) to explore any major structural difference at the grain fragment level and starch granule level. To solve the challenge of selecting the representative sample, a random micrograph from many micrographs with similar features and appearance was selected as a representative picture.

3. Results and Discussion

The SEM images for steam pelleted sorghum and barley samples are shown in Figure 1 and 2, respectively. These images show the difficulty of characterizing grain fragment borders in the pellet due to the union of fragments caused by the high compaction force between particles and the die surface during pelleting process. Union of grain fragments is considered extremely important in determining pellet quality (pellet durability) which has been reported to be related to the extent of starch gelatinization which enhances the binding properties of grain fragments (Thomas *et al.,* 1996). When starch granules in pelleted grains are compared with unprocessed starch granules in sorghum (Figure 3) and barley grains (Figure 4), it seems that there was no swelling in starch granules after pelleting process has occurred. This may be due to low level of moisture addition during pelleting process. From a process prospective, Leaver (1988) reported that the maximum inclusion level of water during the pellet process should not exceed 6%. It has been reported that water addition above this level can cause die blockage and increase the energy required for the pelleting process (Thomas *et al.,* 1996). Svihus *et al*. (2005) reported that the extent of starch gelatinization that occurs after pelleting process ranges from 1-20% of the total starch content. Stevens (1987) reported that conditioning the mash corn caused a limited gelatinization and that most of the starch gelatinization occurred when the feed material passed through the die.

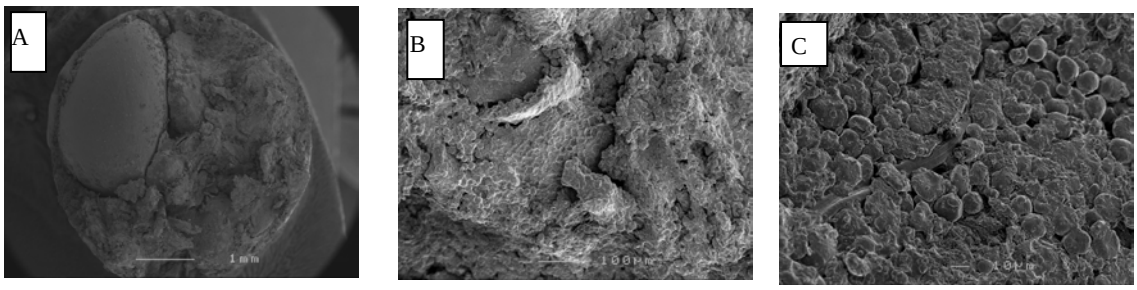

Figure 1. SEM image for sorghum pellets at different magnification levels Figure 1A show cross section of sorghum pellet. Figure 1B and 1C shows no changed occurred to starch granules after pelleting process.

These findings may suggest that starch gelatinization occurs on pellet surface when the feed material pass through the die which keeps the integrity of pellet after pressing through the die.

The SEM images of extruded sorghum and barley are shown in Figure 5 and Figure 6, respectively. The SEM images provide a clear view of changes of the intact starch granules in the extruded sorghum and barley samples. In raw unprocessed.

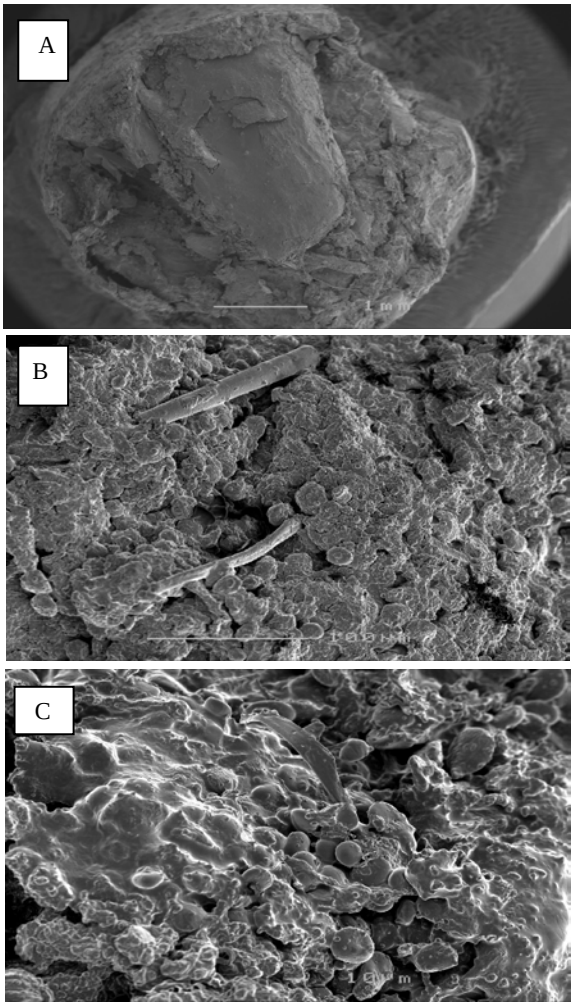

Figure 2. SEM image for barley pellets at different magnification levels. Figure 1A show cross section of barley pellet. Figure 1B and 1C shows no changed occurred to starch granules after pelleting process.

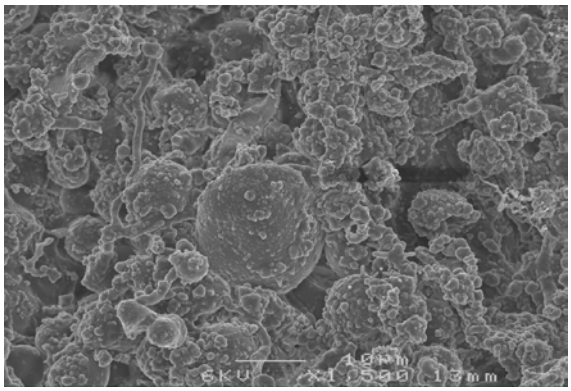

Figure 3. SEM image for raw unprocessed sorghum grain.

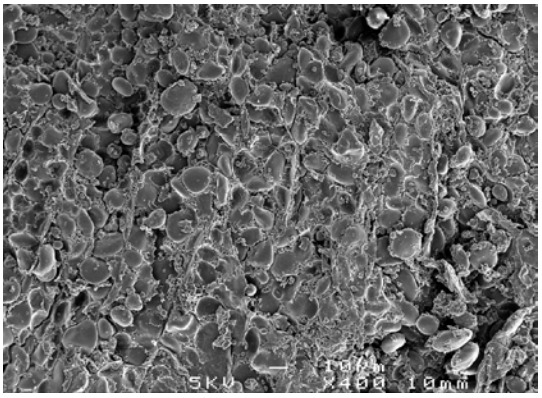

Figure 4. SEM image for raw unprocessed barley grain

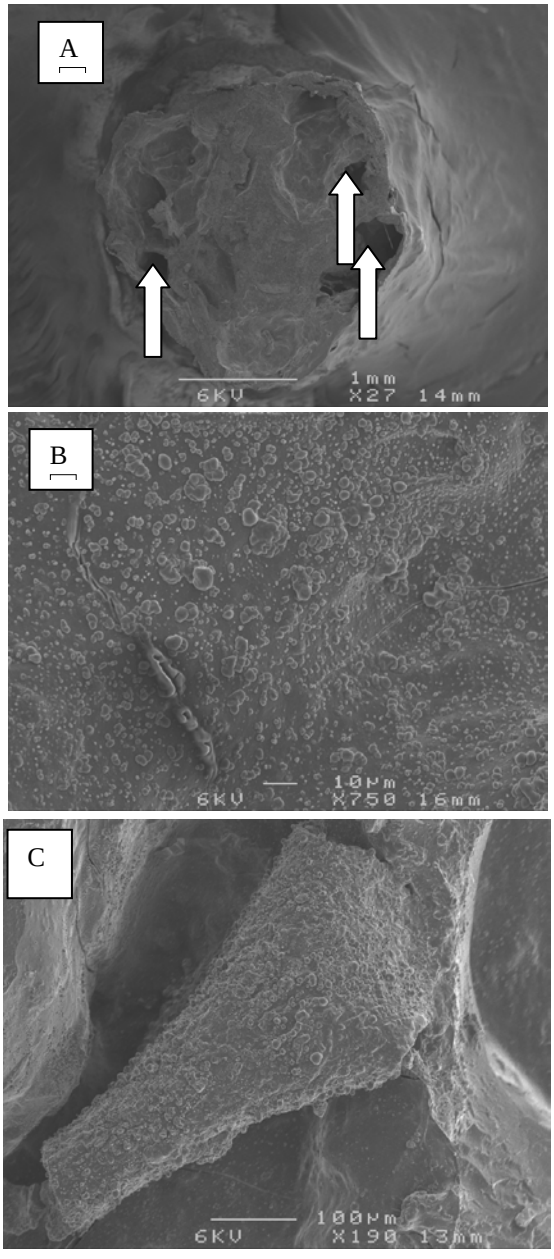

Figure 5. SEM image for sorghum extrudate at different magnification levels. White arrows in Figure 5A show formation of air sacs after extrusion process.

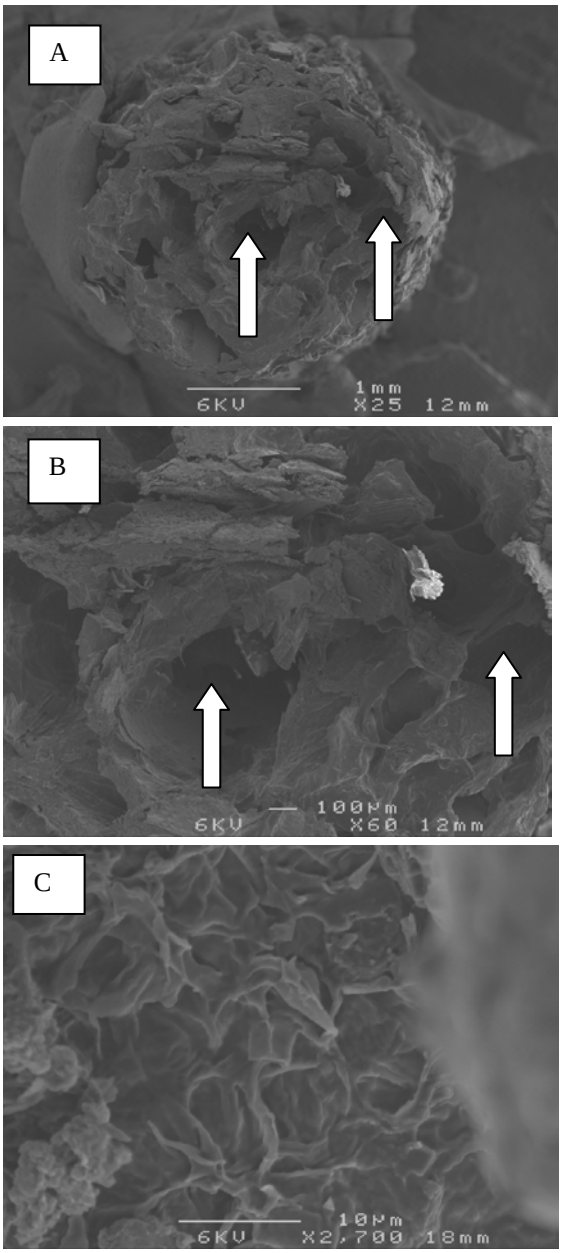

Figure 6. SEM image for barley extrudate at different magnification level. White arrows in Figure 6A and 6B indicate formation of air sacs.

grains, SEM images for sorghum (Figure 3) and barley grains (Figure 4) show clearly the oval-shaped and circular starch granules and protein matrix that is attached to starch granules. However, the extrusion process for both grain types resulted in swelling and melting of starch granules and consequently changed the microstructure of starch granules of being shapeless and mud-like structure as described previously by Srikaeo (2008) and to scatter protein matrix that surround starch granules as shown clearly and more pronounced in sorghum extruded samples micrograph (Figure 5-C). Due to high shearing force and thermal temperature, an extrusion process has been reported to achieve complete starch gelatinization and to completely rupture starch granules (Skoch *et al.*, 1983). SEM images of cross sections of sorghum and barely extrudate showed the presence of small numbers of air sacs of irregular size (Figure 5-A and Figure 6-A, respectively). These air sacs resulted from the rapid reduction in pressure once extrudate exposed to atmospheric pressure and consequently rapid evaporation of internal moisture (Berrios *et al.*, 2004).

In comparison to extrusion process, commercial processes (such as pelleting) are usually operated under less energy-intensive conditions and thus are considered as less expensive compared to extrusion process. In addition, nutrient digestibility (such as protein and starch digestibility) after extrusion process seems to be grain dependent (Ezeogu *et al.*, 2005). For example, exposing sorghum to high thermal treatments has been reported to form disulphide bond cross-linked prolamin proteins and extensive polymerisation of the prolamins which limit protein digestibility (Ezeogu *et al.*, 2005). The presence of tight protein matrix that surrounds starch granules within densely packed endosperm cells in sorghum can also reduce the extent of starch digestion (Hamaker *et al.*, 1987; Rooney and Pflugfelder, 1986). A recent study showed that the extant and the rate of starch digestibility were higher in barley extruded grains compared to sorghum grains (Al-Rabadi *et al.*, 2009). These factors may make the extrusion process as not the best processing method to adopt in feed industry for sorghum grains.

4. Conclusion

It can be concluded from micrograph images that starch granules in both grain types retain its integrity after the pelleting process. However, exposing grain fragments of barley and sorghum to extrusion process can eliminate the integrity of starch granules by inducing swelling and melting of starch granules. Scanning electron microscopy could be practical for feed industries for the quality control in cereal based diets where starch gelatinization is the most influential factor on starch digestibility.

References

Al-Rabadi GJ, Gilbert RG and Gidley MJ. 2009. Effect of particle size on kinetics of starch digestion in milled barley and sorghum grains by porcine alpha-amylase. *J Cereal Sci*, **50**: 198–204.

Ballogou VY, Dossou J, Comlan A, Souza de. 2011. Controlled Drying Effect on the Quality of Sorghum Malts Used for the Chakpalo Production in Benin. *Food Nutrition Sci.,* **2**: 156-161

Berrios J, Wood DF, Whitehand LW, and Pan J. 2004. Sodium bicarbonate and the microstructure, expansion and color of extruded black beans. *J Food Processing and Preservation*, **28**: 321–335.

Gilpin, AS, Herman TJ, and Fairchild FJ. 2002. Feed moisture, retention time, and steam as quality and energy utilization determinants in the pelleting process. *Appl Engineering in Agri.,* **18**: 331-338.

Ezeogu LI, Duodu KG and Taylor, JRN. 2005. Effects of endosperm texture and cooking conditions on the in vitro starch digestibility of sorghum and maize flours. *J Cereal Sci.,* **42:** 33–44.

Hamake, BR, Kirleis AW, Butler, LG, Axtell JD and Mertz ET. 1987. Improving the in vitro protein digestibility of sorghum with reducing agents. *Proc Natl Acad Sci. U. S. A.* **84**: 626–628.

Rooney LW and Pflugfelder RL. 1986. Factors affecting starch digestibility with special emphasis on sorghum and corn. *J Anim Sci.,* 63: 1607–1623.

Leaver, RH., 1988. **The Pelleting Process.** Sprout-Bauer, Muncy, PA.

Lee EY, Lim K, Lim JK and Lim ST. 2000 Effects of gelatinization and moisture content of extruded starch pellets on morphology and physical properties of microwave-expanded products. *Cereal Chem*., **77**: 769–773.

Olav FK and Svihus B. 2011. Tools to Determine the degree of starch gelatinization in commercial extruded salmon feeds. *J World Aquaculture Soc*., **42**: 914–920.

Skoch ER, Binder SF, Deyoe CW, Allee GL, and Behnke KC. 1983. Effects of steam pelleting conditions and extrusion cooking on a swine diet containing wheat middlings. *J Anim Sci.,* **57**: 929–935.

Srikaeo K. 2008. Microscopy and image analysis techniques for quality control in food industry: A Case study of wheat grain cooking process. *J Microscopy Soc Thailand,* **22**: 46-49.

Srikaeo K, Furst, JE, Ashton JF and Hosken RW. 2006. Microstructural changes of starch in cooked wheat grains as affected by cooking temperatures and times. *LWT*. **39**: 528-533.

Stevens CA. 1987. Starch gelatinization and the influence of particle size, steam pressure and die speed on the pelleting process. Doctoral Dissertation. Kansas State University, Manhattan, Kansas.

Svihus B, Kløvstad KH, Perez V, Zimonja O, Sahlstrom S, Schüller RB, Jeksrud WK and Prestløkken, E. 2004. Physical and nutritional effects of pelleting of broiler chicken diets made from wheat ground to different coarsenesses by the use of roller mill and hammer mill. *Anim Feed Sci Technol.,* **117:** 281-293.

Thomas M and van der Poel.AFB. 1996. Physical quality of pelleted animal feed: 1.Criteria for pellet quality. *Anim Feed Sci Technol.,* **61**:89–112.

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. 4399
Fax no. : 0096253903349
E. mail: jjbs@hu.edu.jo

Hashemite Kingdom of Jordan

The Hashemite University

المجلة الأردنية للعلوم الحياتية

مجلة علمية عالمية محكمة

تصدر بدعم من صندوق دعم البحث العلمي

ISSN 1995-6673

المجلة الأردنية للعلوم الحياتية
مجلة علمية عالمية محكمة

المجلة الأردنية للعلوم الحياتية : مجلة علمية عالمية محكمة ومفهرسة ومصنفة، تصدر عن الجامعة الهاشمية و بدعم من صندوق دعم البحث العلمي- وزارة التعليم العالي و البحث العلمي .

هيئة التحرير

فريق الدعم:

ترسل البحوث إلى العنوان التالي :

رئيس تحرير المجلة الأردنية للعلوم الحياتية
عمادة البحث العلمي و الدراسات العليا
الجامعة الهاشمية
الزرقاء – الأردن
هاتف : ٣٩٠٣٣٣٣ ٥ ٠٠٩٦٢ فرعي ٤١٤٧
Email: jjbs@hu.edu.jo, Website: www.jjbs.hu.edu.jo