المجلة الأردنية للعلوم الحياتية
Jordan Journal of Biological Sciences (JJBS)

http://jjbs.hu.edu.jo

Jordan Journal of Biological Sciences (JJBS) (ISSN: 1995–6673): 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Manuscript, or the essence of their content, must be previously unpublished and should not be under simultaneous consideration by another journal.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, Number2, June 2014
ISSN 1995-6673

Jordan Journal of Biological Sciences

CONTENTS

JJBS

Volume 7, Number 2, June .2014
ISSN 1995-6673
Pages 81 - 94

Jordan Journal of Biological Sciences **Review**

Clostridium: Pathogenic Roles, Industrial Uses and Medicinal Prospects of Natural Products as Ameliorative Agents against Pathogenic Species

Sabina M. Num[1] and Nicodemus M. Useh [2, 3,*]

1Department of Veterinary Pathology and Microbiology, University of Agriculture, Makurdi,

2Department of Veterinary Pathology and Microbiology, Ahmadu Bello University, Zaria, Nigeria;

3 Department of Population Medicine and Diagnostic Sciences, College of Veterinary Medicine, Cornell University, Ithaca, New York, United States of America

Received: November 30, 2013 Revised: January 4, 2014 Accepted: January 13, 2014

Abstract

The genus *Clostridium* is made up of species that cause disease to both human beings and animals, with zoonotic species/strains playing critical roles in disease dynamics. Clostridial organisms posses pathogenic, therapeutic and industrial uses. Pathogenic clostridia cause lethal or life threatening infections that must be treated, while the industrial clostridia produce bio-fuels, to serve as alternative energy sources in the face of very high global prices of conventional fuels. Genetic engineering is currently developing novel strains by replacing or altering the gene configuration of pathogenic strains (gene knockout/down) to convert them to solvenogenic and non pathogenic strains for industrial uses. Therapeutic clostridia serve as vehicles for treatment of diseases, especially solid tumors. Because of the global problem of antibiotic resistance, which is a survival strategy by microbial pathogens, some of the well known chemotherapeutic protocols have failed and there is the need to evolve new treatment approaches, using novel agents that have superior mechanisms of action, compared to conventional antibiotics that are becoming inconsequential because of resistance. It is believed that natural products may be effective alternatives to resolving this puzzle, since they are cheaper, readily available and possibly effective against clostridial species. In this review article, the authors discussed some of the life threatening and solvenogenic clostridia and listed some natural products that may possibly be employed as drug targets for ameliorating the problem of resistance in the future, if their active principles are thoroughly researched. It is concluded that medical and veterinary research should be re-jigged to evolve active principles from natural products that are not so easily surmounted by the surge in drug resistant strains.

Keywords: *Clostridium*; Industrial Uses; Medicinal Prospects; Natural Products; Ameliorative Agents; Pathogenic Species..

1. Introduction

The genus *Clostridium* consists of over 100 species, ranking second in size next to Streptomyces (Dong *et al.*, 2010). Many *Clostridium* species are known to cause disease to human beings. These include neurotoxigenic clostridia (*C. botulinum* and *C. tetani*) (Weingart *et al.*, 2010), clostridia involved in gas gangrene and necrotizing infections (*C. perfringens, C. sordellii* and *C. septicum*) (Hatheway, 1990; Aldape *et al.*, 2006), and the enteropathogenic *C. difficile* (Twine *et al.*, 2009). *Clostridium chauvoei*, which has a strong phylogenetic relationship with *C. septicum*, a human pathogen, has a long history of veterinary importance. It was believed for a very long time that this species was exclusively a veterinary pathogen, only associated with blackleg in ruminants (Useh *et al*., 2006a). Recent reports of the disease in human beings have placed the pathogen on the list of very important lethal disease agents of human beings (Nagano *et al.*, 2008; Weatherhead and Tweardy, 2011). Indeed zoonotic strains of the *Clostridium* spp pose a great threat to community health in endemic areas. The aforementioned notwithstanding, genetic engineering of pathogenic strains is beginning to suggest that members of the genus could also serve therapeutic purposes. In tumor managements, the use of viral vehicles in gene therapy could be successful or not, depending on the delivery systems employed. There are many tumors in

* Corresponding author. e-mail: nicodemus.useh@fulbrightmail.org.

which this procedure has not been successful. Thus, there is intensive on-going research in so many expert laboratories to develop alternative management approaches (Minton, 2003). On the other hand, some *Clostridium* species are of great industrial importance. For example, *C. thermocellum* can produce ethanol from lignocellulosic waste at high temperature, while *C. acetobutylicum* and *C. beijerinckii* produce solvents (acetone, butanol, and ethanol) by utilizing a variety of substrates from monosaccharides to polysaccharides (Ezeji *et al.,* 2007; Otte *et al.,* 2009; Ezeji *et al*., 2010; Jia *et al*., 2011).

2. 2. Pathogenic Clostridia

2.1. Clostridium Difficile

Clostridium difficile is the most common cause of infectious diarrhoea in hospitalized patients in the industrialized world (Johnson and Gerding, 1998). With symptoms ranging from self-limited diarrhoea to life threatening fulminant colitis, *C. difficile* infection (CDI) has affected hundreds of thousands of patients worldwide and substantially burdens health care resources (Kyne *et al.,* 2002). In particular, CDI has caused increased patient morbidity and mortality in hospitals throughout the world since 2003, when outbreaks with increased disease incidence and severity first emerged (Forgetta *et al*., 2011), and from 2004 to 2007 contributed to almost 1,000 deaths in the province of Quebec, Canada (Gilca *et al*., 2008 cited by Gilca *et al*., 2011). Investigations of these and other outbreaks across Canada, the United States, and Western Europe led to the recognition of a severe-disease associated (SDA) strain predominantly responsible for this epidemic (Kuijper *et al.,* 2006). This strain has been classified as North American pulse field type 1 (NAP1), ribotype 027, toxinotype III, or restriction-endonuclease type BI. Outbreaks have also been associated with other SDA strains, such as the NAP7/toxinotype V/ribotype 078 (NAP7) strain found in cases of human and animal disease (Goohuis *et al.,* 2008; Mulvey *et al.,* 2010) and multiple toxin A-negative B-positive (A-B+) pulsotypes and ribotypes responsible for CDI outbreaks in Ireland, the United Kingdom, the United States, and Canada (Al-Barrack *et al*., 1999; Stabler *et al*., 2006). *Clostridium difficile* is the leading cause of health care–associated infectious diarrhoea. After exposure to *C. difficile*, some patients remain asymptomatic, whereas others have illness ranging from mild diarrhoea to fulminant colitis (Johnson and Gerding, 1998). Outbreaks of *C. difficile* infection in North America and Europe have been attributed to the emergence of an epidemic strain (North American pulsed-field gel electrophoresis [PFGE] type 1 [NAP1]) (McDonald, 2005).

Clostridium difficile is the most commonly identified bacterial cause of HIV-related and all nosocomial diarrhoea (Bartlett, 2007). Whereas outbreaks in North American and European hospitals have led to increased *C. difficile* infection (CDI) vigilance in developed countries, data on its significance for developing countries is scarce (Warny *et al*., 2005). This information gap is significant since antibiotic pre-exposure, an important CDI risk factor, is high in many developing countries due to unregulated access (Sosa, 2005). Recent data suggest CDI is under-recognized in Latin America (Legaria *et al.,* 2003). Data from sub-Saharan Africa is sparse. Despite liberal, unregulated antibiotic use, developing countries do not have data about *C. difficile* infection. Nigeria, Africa's most populous country, has the third highest absolute HIV burden worldwide. Over-the-counter antibiotic access is widespread in Nigeria and nosocomial diarrhoea incidence is high (Nwokediuko *et al.,* 2002). However, patients are not tested for CDI due to presumptions of low prevalence and technical incapacity. Result from the study by this group showed significant CDI in patients with HIV infections in a 5 star Hospital in Nigeria.

C difficile has been reported as a pathogen of animals, affecting dogs, cats, horses and pigs (Weese *et al*., 2001a,b; Arroyo *et al*., 2005; Keel *et al*., 2007; Keessen *et al*., 2010, 2011). In one study, some authors reported *C. difficile* colonization in dogs and cats in a longitudinal manner, indicating that *C. difficile* is commonly albeit sporadically found in the faeces of healthy pets (Weese *et al*., 2010). Another study in the Netherlands showed high *C. difficile* prevalence in dogs, cats, horses, pigs, poultry and calves. Twenty-two different PCR ribotypes were identified and all *C. difficile* isolates from pigs, cattle and poultry were toxinogenic, whereas the majority of isolates from pet animals consisted of non-toxinogenic PCR ribotypes 010 and 039. Ribotype 012 was most prevalent in cattle and ribotype 078 in pigs. No predominant ribotypes were present in horse and poultry samples. Overall, PCR ribotypes 012, 014 and 078 were the most frequently recovered toxinogenic ribotypes from animal samples. Comparison with human isolates from the Dutch Reference Laboratory for *C. difficile* at Leiden University Medical Centre (LUMC) showed that these types were also recovered from human hospitalized patients in 2009/2010, encompassing 0.8%, 11.4% and 9.8% of all isolates, respectively. Application of multiple locus variable-number tandem-repeat analysis indicated a genotypic relation of animal and human ribotype 078 strains, but a clear genotypic distinction for ribotypes 012 and 014. Contrary to PCR ribotype 078, significant genetic differences were observed between animal and human PCR ribotype 012 and 014 isolates. The authors concluded that toxinogenic *C. difficile* PCR ribotypes found in animals correspond to PCR ribotypes associated with human disease in hospitalized patients in the Netherlands (Koene *et al*., 2011).

It is believed that *C. difficile* is the most important cause of outbreaks of neonatal diarrhoea in pig husbandry (Songer and Uzal, 2005). Infection of neonatal piglets occurs by environmental transmission (Hopman *et al.,* 2010). The bacteria have also been detected in air samples, but the role of aerial dissemination in the transmission of *C. difficile* to piglets has yet to be conclusively elucidated (Hopman *et al.,* 2010). *C. difficile* has also been reported in retail meat in Sweden (von Abacron *et al.,* 2009), broiler chickens sold at market places in Zimbabwe (Simango and Mwakurudwa, 2008), chicken in retail poultry meat

in the United States of America (Songer *et al*., 2009; Harvey *et al*., 2011a,b), retail vegetables in Canada (Al-Salif and Brazier, 1996; Bakri, 2009; Metcalf *et al*., 2010) with a zero prevalence in passerine birds in Europe (Bandelj *et al*., 2011). The meaning of all these reports is that *Clostridium* as an organism is set to colonize the entire environment to create havoc to public health.

In the light of the ongoing uncertainty about the zoonotic potential of *C. difficile* and the emergence of hypervirulent strains, research on the possible routes of transmission from animals to human beings and between animals is becoming increasingly important. There is still little knowledge on possible transmission routes from animals to human beings (Jhung *et al.,* 2008). Possible routes other than meat consumption (direct and indirect contact) with animals, have been speculated (Weese, 2010). It is increasingly recognized that aerial dissemination of bacterial elements can be the main route of zoonotic transmission (Kuske, 2006; Keessen *et al*., 2011).

2.2. Clostridium Chauvoei

Blackleg in ruminants has been reviewed (Useh *et al.,* 2006a). It is a fatal disease of cattle and sheep mainly (but has also been reported in deer and other animal species), caused by *C. chauvoei*, and was first reported in 1870 (Armstrong and McNamee, 1950). Other synonyms of the disease are: black quarter, emphysematous gangrene, symptomatic anthrax, quarter ill (Merchant and Barner, 1964), gangrenous myositis (Williams and Andrews, 1992), clostridial myositis of ruminants (Williams, 1977) and clostridial myocarditis (blackleg of the heart) (Uzal *et al*., 2003). *C. chauvoei* was named after Professor J. A. B. Chauveau, a French bacteriologist (Cato *et al*., 1986), and 23 reference strains of the bacterium have been described (Holderman *et al.,* 1977). In Nigeria, Jakari, Vom and K76 strains have been incriminated as the aetiologic agents of the disease (Bagadi, 1977), while NCTC 8076 and 8361 (Heurmann *et al.,* 1991), strain 49 (Singh *et al*., 1993), strain NC 08596 (Heurmann *et al.,* 1991), strains ch.16 and ch.22, strain Tukyu and strain Awasa (Mhoma *et al.,* 1995) have been reported to cause blackleg in UK, India and other parts of Asia, Germany, other parts of Europe and the Americas, Mozambique, Tanzania and other parts of east Africa and Ethiopia, respectively. These strains are also responsible for the disease in both the natural and accidental hosts (Radostits *et al.,* 2000).

2.3. Clostridium Botulinum

Botulinum neurotoxins are 150-kDa endopeptidase toxins that are produced by C. *botulinum, C. butyricum* and *C. baratii* (CDC, 1998). These neurotoxins are considered the most toxic substances known (Lamanna, 1959) and have been designated as category A biological threat agents (Rotz *et al.,* 2002). Botulism, a serious neuroparalytic and sometimes fatal illness, caused by potent neurotoxins produced by the gram-positive, anaerobic, spore-forming bacterium *C. botulinum*, affects both animals and human beings. Outbreaks have been reported following consumption of unsafe canned vegetables (Date *et al*., 2011). Rodloff and Krüger (2012) reviewed the occurrence of visceral botulism in farmers. Seven types of botulinum toxins are known (A through G), of which types A, B, E, and F cause virtually all cases of human botulism (CDC, 1998). Botulism is characterized by rapidly progressive cranial neuropathy and symmetric descending flaccid paralysis, which may progress to respiratory arrest requiring mechanical ventilation and intensive supportive care in 60% of patients and clinical recovery takes several weeks to months (Shapiro *et al.,* 1998). Botulism neurotoxin derivative has been engineered to allow self activation (Masuyer *et al.,* 2011).

2.4. Clostridium Septicum

The organism is the causative agent of muscular gangrene syndrome with clinical features that overlap with those of other clostridial diseases and acute/hyperacute syndromes seen with other diseases such as anthrax (Fasanella *et al.,* 2010). *C. septicum* and *C. chauvoei* have a close phylogenetic relationship that made science unable to distinguish the two organisms convincingly for a very long time (Nagano *et al*., 2008). Traditional microbiological methods are unable to distinguish the two microbial species. Molecular diagnostics, based on real time PCR with primers designed according to sporulation, 16s rRNA and triose pentose isomerase gene sequences have been reported (Lange *et al*., 2010; Ham *et al*., 2010; Garofolo *et al*., 2011). Soft tissues are usually affected in the pathology of the disease with an accompanying necrotizing infection (Gnerlich *et al.,* 2011; Kiel *et al*., 2011).

2.5. Clostridium Perfringens

C. perfringens is a Gram-positive rod, spore-forming, anaerobic bacterium, which is highly associated with a wide array of gastrointestinal (GI) and histotoxic diseases in both humans and animals (McClane, 2007; Amimoto *et al.,* 2007; Keyburn *et al.,* 2008). The most common cause of *C. perfringens*-associated food poisoning in humans is the consumption of *C. perfringens* vegetative cells followed by sporulation in the gut (Paredes-Sabja and Sarker, 2009). The early steps in the development of *C. perfringens*-associated gas gangrene and other non-food-borne GI illnesses, such as antibiotic-associated diarrhoea, occur when *C. perfringens* spores ubiquitously found in the environment come in contact with the host, where spores undergo germination followed by outgrowth, cell proliferation and toxin secretion (Paredes-Sabja and Sarker, 2012).

3. Non-Pathogenic Clostridia

The biofuel producing clostridia are very important as they are nonpathogenic and produce biogenic fuels for industrial use (Köpke *et al.,* 2011). For instance, 2,3-Butanediol (2,3BD) is a commodity chemical usually produced from oil. It can be used as a precursor in the manufacture of a range of chemical products, including the solvents methyl ethyl ketone (MEK), gamma-butyrolactone (GBL), and 1,3-butadiene. Commercially, the key downstream products of 2,3BD have a potential

global market of around 32 million tons per annum, valued at approximately $43 billion in sales (Xiu and Zeng, 2008). Three (3) acetogenic members of the *Clostridium* genus (*C. autoethanogenum, C. ljungdahlii*, and *C. ragsdalei*) have been reported to produce 2,3 BD using gases (carbon monoxide [CO] or hydrogen [H_2] and CO) as the source of carbon and energy. 2,3-butanediol (2,3BD) is a high-value chemical usually produced petrochemically but can also be synthesized by some bacteria. To date, the best microbial 2,3BD production rates have been observed using pathogenic bacteria in fermentation systems that depend on sugars as the carbon and energy sources for product synthesis. Homologues of the genes involved in the requisite pathway have been identified (Köpke *et al*., 2011). In the report, a gene expression study demonstrated a correlation between mRNA accumulation from 2,3BD biosynthetic genes and the onset of 2,3BD production, while a broader expression study of Wood-Ljungdahl pathway genes has been shown to provide a transcription-level view of one of the oldest existing biochemical pathways. Genetic engineering is in its top form to develop new stains of clostridial organisms with non-pathogenic factors and great industrial uses (Zhang *et al*., 2012).

Acetone-butanone-ethanol (ABE) fermentation involves the production of these via fermentation. This is re-invigorated by the high and ever rising cost of crude and the desire for alternative energy sources to cut cost. As a result of increasing oil prices and extensive oil consumption accompanied by the potentially serious environmental impacts of oil extraction, the use of renewable biofuels as a partial replacement for traditional fossil fuels has gained recent worldwide attention. Among all of the alternatives, butanol obtained from the acetone-butanol- ethanol (ABE) process through a biological approach has become a potential replacement fuel (Yen *et al*., 2011; Bankar *et al*., 2011; Nakayama *et al*., 2011; Survase *et al*., 2011; Jones *et al.,* 2011; Yu *et al.,* 2011). The shortage of gasoline, even in some oil producing countries, has stimulated governments of countries like China to re-awaken their biofuel programme (Dong *et al*., 2011). Clostridial organisms such as *C. acetobutylicum, C. beijerinckii, C. saccharobutylicum*, and *C. saccharoperbutylacetonicum* are known for producing biofuels (Jones and Kreis, 1995). Genetic manipulation of these bacteria through gene knockout/ down systems have been developed through homologues recombination methods based on replicable/ non replicable vectors (Harris *et al*., 2002; Tomas *et al*., 2004; Heap *et al*., 2007; Papoutsakis, 2008; Heap and Minton, 2009; Kuehne *et al*., 2011; Lütke-Eversloh and Bahl, 2011). Antisense RNA strategies have also been employed to manipulate the genetic code of clostridia for biofuel production (Desai and Papoutsakis, 1999; Tummala *et al.,* 2003). *C. acetobutylicum* entire genome has been sequenced in an attempt to identify candidate genes for the purpose of genetic manipulation for biofuel production and other purposes (Bao *et al*., 2011). Biofuel tolerant mutants have been developed to enhance biofuel production by some non-pathogenic clostridia (Mao *et al.,* 2011). Apart from the usefulness of non-pathogenic clostridia in biofuel production, it is believed that their spores could serve as vehicles for systematic colonization of tumor cells in cancer therapy (Fox *et al*., 2000; Barbe´ *et al.,* 2006).

4. Clostridial Organisms as Agents of Bioterrorism

Clostridial organisms are targets for bioterrorism in our changing world where microorganisms have been used as agents of biological attack. Botulinum toxin in bioterrorism has been reviewed (Bossi *et al*., 2006). Aerosols of botulinum toxin could be used as a biological weapon (Franz *et al.,* 1997; Lecour *et al.,* 1998; Arnon *et al*., 2001). Deliberate release may also involve contamination of food or water supplies with toxin or *C. botulinum* bacteria. Botulinum toxin is extremely lethal and easy to produce. The Aum Shinrikyo cult in Japan attempted unsuccessfully to release an airborne form of botulinum toxin in Tokyo on three separate occasions in the early 1990s (Arnon *et al*., 2001). It is likely that several countries have developed and stockpiled botulinum toxin weapons (Arnon *et al.,* 2001). It has been estimated that a point-source aerosol release of botulinum toxin could incapacitate or kill 10% of the population. *C. botulinum* is a large, Gram-positive, strictly anaerobic bacillus that forms a subterminal spore. These spores can be found in soil samples and marine sediments throughout the world. Four groups of *C. botulinum* are described. Group I organisms are proteolytic in culture and produce toxin types A, B or F; group II organisms are nonproteolytic and produce toxin types B, E or F; group III produces toxin types C or D, and group IV toxins type G. These toxins are proteins of approximately 150 kD molecular weight and induce similar effects whether inhaled or ingested. When ingested, toxins are absorbed in the duodenum and jejunum, and enter into the bloodstream, and eventually reach peripheral cholinergic synapses. Botulinum toxin does not penetrate intact skin (Arnon *et al.,* 2001). Toxins act by binding to the presynaptic nerve terminal at the neuromuscular junction and at cholinergic autonomic sites. This binding prevents release of acetylcholine and interrupts neuro-transmission (Humeau *et al*., 2000). Human botulism is almost always caused by toxin types A, B, E and, in rare cases, F. Types C and D are associated with disease in birds and mammals. Type G is not associated with any disease in humans or animals. It has been estimated that, weight for weight, these toxins are the most toxic compounds known, with an estimated toxic dose, for toxin type A, of only 0.001 µg/kg of body weight when administered intravenously, subcutaneously or intraperitoneally (Middlebrook and Franz, 1997). By inhalation, the dose that would kill 50% of exposed persons (LD50) is 0.003 µg/kg of body weight. This toxin is 100,000 times more toxic than sarin gas (Fanz *et al*., 1997).

C. botulinum has been classified as a category A biological warfare agent (Johnson and Bradshaw, 2001). There are seven serologically distinct serotypes of the neurotoxin secreted by this bacterium (A–G), with types

A, B, E, and rarely F responsible for human intoxication. Botulinum neurotoxin (BoNT) A is the deadliest of the seven toxins with potency approximately one million times greater than cobra toxin, and one hundred billion times greater than cyanide. The LD50 for humans is approximately 1 ng/ kg of body weight (Schantz and Johnson, 1992). Eubanks *et al.* (2007) outlined the detailed technological advancement for the detection and protection against botulism as a biological warfare agent.

C. perfringens epsilon toxin, has been classified as category B agents (http://www.niaid. nih.gov/ biodefense/bandc_priority.htmj for classification of bio-threat agents) (Marks, 2004). Both *C. perfringens* spores and toxins have reportedly been considered as biological warfare agents (Mangold and Goldberg, 1999; Butler, 1999). Like *B. anthracis* spores, the spores of *C. perfringens* are robust and heat stable, but unlike *B. anthracis* spores there is no evidence that the spores can be delivered by the airborne route to cause disease. Rather, the spores have been considered as infective agents in weapons which cause traumatic injury. Flechette (shrapnel) weapons developed by Japan during World War II resulted in the delivery of spores into wounds and the subsequent establishment of gas gangrene (Mangold and Goldberg, 1999). The treatment of gas gangrene is notoriously difficult, because the infection becomes established in tissues that are deprived of blood. Evidence that the toxins produced by *C. perfringens* are potential biological warfare agents is still a course of dispute. However, it is believed that Iraq had a strong interest in *C. perfringens* toxins as biological weapons (Butler, 1999) and *C. perfringens* toxin is now placed on the list of CDC select agents as a toxin of particular concern. This toxin is active in a wide range of animal species and plays a central role in enterotoxaemia of sheep and lambs (Titball, 2009).

5. Epidemiology and Economic Importance of Clostridial Infections

Clostridium, a eubacteria, consists of diverse species whose economic importance includes the ability to cause disease (pathogenic strains), industrial uses and therapeutic potentials. The pathogenic strains which cause very lethal infections include *C. difficile, botulinum, perfringens, septicum, chauvoei*, etc. Clostridial infections cut across animal and human populations, with zoonotic species/ strains posing a great threat to community health in endemic areas. *C. difficile* infection is an increasing burden to the health care system, totaling more than $1 billion/ year in the United States of America alone (Leffler and Lamont, 2009).

Life-threatening soft tissue infections caused by *Clostridium* species have been described in the medical literature for hundreds of years largely because of their fulminant nature, distinctive clinical presentations and complex management issues. *C. perfringens*, *C. septicum* and *C. histolyticum* are the principal causes of trauma-associated gas gangrene and their incidence increases dramatically in times of war, hurricanes, earthquakes and other mass casualty conditions (Stevens *et al.*, 2011). Currently, tetanus occurs in about 1,000,000 people annually worldwide, and most patients are newly born in developing countries located in tropical areas. The World Health Organization (WHO) estimated that there were 715,000 deaths from tetanus among newborns in 1990. Neonatal tetanus is considered endemic in 90 developing countries and resulted in 248,000 deaths in 1997 (World Health Organization; http://www.who.int/vaccine-diseases/ NeonatallTetanus.shtml). Tetanus continues to cause ~250,000 deaths worldwide each year, predominantly in low- and middle income countries (Alam *et al*., 2008).

The life threatening consequences of clostridial organisms have been reviewed (Stevens *et al.*, 2011). During the civil war, 50% of soldiers who were wounded ultimately died. If the soldier was not killed outright, the extensive destruction of tissue inflicted by the 45 caliber lead bullets used at the time predisposed him to the development of gas gangrene, largely due to the resultant vascular damage. Civil war surgeons had few resources such as anaesthesia, intravenous fluids and antibiotics and thus “prophylactic amputation” became common place. During World War I, medical treatments had improved. However, weaponry also became more sophisticated. In this period, the incidence of gas gangrene was as high as 10% of wounded soldiers (MacLennan, 1962). Gas gangrene also claimed an estimated 100,000 German soldiers during this period.

In World War II, medical evacuation, improved surgical debridement techniques in field hospitals, gas gangrene anti-toxin and antibiotics were available and reduced the overall incidence of gas gangrene. For example, death from gas gangrene in US casualties in the western front of Europe was 8 cases/1000 wounded, for the Free French Forces it was 12.3/1000 and among prisoners of war, 51.9/1000 wounded. The much higher rate among prisoners was attributed to a delay in definitive surgical treatment which was 1 day for US casualties and 3.5 days for prisoners (MacLennan, 1962). In addition, the incidence of gas gangrene was higher in European theaters than in desert operations, likely because the fertile valleys of Europe were heavily contaminated with *C. perfringens* (50,000 per gram of soil), whereas they were rarely recovered in the Sahara Desert. Uniforms too were found to be contaminated with enteric clostridia and *C. perfringens* was isolated from 24% of shirts and 44% of trousers (McLennan, 1943a,b,c).

In the Korean, Vietnam, Gulf and Afghanistan wars, rapid evacuation, thorough (but not radical) surgical debridement, vascular reconstruction, improved supportive measures and greater antibiotic efficacy and availability greatly reduced the incidence of *C. perfringens* gas gangrene. Gas gangrene continues to be problematic in both military theaters and the civilian sectors, always associated with traumatic injuries. For instance, among nearly 2800 patients hospitalized following the 2008 Wenchuan earthquake in China, over 2.4% developed gas gangrene (Chen *et al.*, 2011). Interestingly, *C. septicum* as a cause of non-traumatic

gas gangrene has increased in civilian populations in recent years and necrotizing infections associated with skin-popping black tar heroin have been attributed to *C. perfringens, C. novyi* and *C. sordellii. C. sordellii* has been reported not only in trauma cases but also associated with pregnant females undergoing normal vaginal delivery, caesarian sections or medical abortions. *C. difficile*, the causative agent of antimicrobial- associated diarrhoea and pseudo-membranous colitis, is currently one of the most important nosocomial pathogens because of its zoonotic nature (Rupnik *et al*., 2009). Nosocomial infections with the Gram-positive pathogen *C. difficile* pose a major risk for hospitalized patients and result in significant costs to health care systems.

Blackleg is known to be an endemic disease in both developed and developing countries of the world and is a well-known cause of financial loss to cattle raisers in many parts of the world (Adams, 1998). The economic losses of ruminants to the disease have yet to be quantified in most countries, but in Nigeria, losses of Zebu cattle to the disease have been estimated at US$4.3 million annually (Useh *et al*., 2006b). In United States of America, Latin America, India and other parts of Asia and Europe, the economic losses of ruminants to blackleg have yet to be estimated, but it has been reported that the disease causes major economic losses in cattle and minor losses in sheep (Cottral, 1978; Ramarao and Rao, 1990; Troxel *et al*., 1997; Flyod, 1994; Adams, 1998).

6. Management, Prevention and Control of Infections by Pathogenic Clostridia

Resistance of pathogenic clostridial organisms to therapeutic antimicrobials has been reported and is increasingly becoming an issue of concern to public health (Gamboa-Coronado *et al.,* 2011). The resistance means that alternative therapeutic protocols will have to be developed in the future to treat resistant strains. Management of *C. difficile* infection in human beings has been reviewed (Leffler and Lamont, 2009). Drugs like metronidazole, (oral administration), vancomycin and several stand-alone therapies or as adjunct agents have been used. Alternative treatments include the use of rifaximin (Johnson *et al.,* 2007; Garey *et al.,* 2008). Immunotherapy (Kyne *et al*., 2001), microbiologic therapy (Seal *et al.,* 1987) and probiotic therapy (McFarland, 2006) have also been carried out. Prevention and control is better achieved by appropriate use of antibiotics, especially broad spectrum agents and those shown to have a particular predisposition for *C. difficile* infection. Treatment of clostridial infections caused by the highly toxigenic strains involves neutralization of the toxins.

The drug of choice for treatment of blackleg is procaine penicillin (10 000 IU/kg) over a 3–5 day therapy period. It is generally advocated that the administration of crystalline penicillins intravenously should precede the long-acting preparations that should be administered directly in the affected tissues (Radostits *et al*., 2000). The best preventive and control strategy is vaccination.

7. Natural Products as Potential Therapeutic Agents Against Clostridial Infections

Globally, millions of people in the developing world rely on medicinal plants for primary health care, income generation and livelihood improvement (WHO, 2002). The active substances present in many medicinal plants could be used as therapeutic alternatives against clostridial infections (Woodford and Livermore, 2009). Although the mechanisms of pharmacological actions of most medicinal plants with potentials to ameliorate clostridial infections have yet to be defined, there are strong indications that the results of most *in vitro* studies that seem to point to some beneficial actions of these plants is worth further investigations after all. Table 1 shows a list of medicinal plants from different parts of the world with potential therapeutic properties against clostridial infections.

C. perfringens is classified into five serotypes (A, B, C, D and E) based on the type of enterotoxins it produces. Delta-toxin is one of the five haemolysins released by *Clostridium perfringens* which play a most important role in gas gangrene and gastroenteritis. The native structure of the delta-toxin is not yet reported in the structural databases (Skariyachan *et al*., 2011). *C. perfringens* has been shown to develop multiple drug resistance, indicating that the treatment for this bacterium is quite challenging. There is need, therefore, to employ the use of alternative therapeutic agents. The rational design of improved therapeutics requires the crystal structure for the toxin. However, the structure for the toxin is not yet available in its native form. In a recent study, some authors modeled the toxin structure using α- haemolysin of *Staphylococcus aureus* (PDB: 3M4D chain A) as template. The docking of the toxin with the herbal extract curcumin (1,7-bis (4-hydroxy-3-methoxyphenyl) hepta-1,6-diene-3,5-dione) showed a binding energy of -8.6 Kcal/mol, in comparison to the known antibiotic linezolid with binding energy of -6.1 Kcal/mol. This data is believed to be relevant in the future design and development of novel compounds against the delta-toxin produced by *C. perfringens*.

Table 1. List of some natural products with ethnopharmacological potentials against clostridial infections

Natural product	possible clostridial target	Reference
Combretum fragrans	*C. chauvoei*	Useh *et al*. (2004)
Aristolochia paucinervis	*C. perfringens/ difficile*	Gadhi *et al*. (1999)
Melia toosendan	*C. botulinum*	Shi & Li (2007);Nakai *et al*. (2009)
Propolis (bee glue)	*Clostridium perfringens*	Boyanova *et al*. (2006)
Clostridium difficile		"
Clostridium tertium		"
Other *Clostridium* species		"
Phyllanthus muellerianus	*C. sporogenes*	Brusotti *et al*. (2011)
Tamarindus indicus	*C. chauvoei*	Useh *et al*. (2004)
Galla Rhois	*Clostridium perfringens, C. paraputrificum*	Ahn *et al*. (1998)
Glycyrrhiza glabra linn	*Clostridium* spp	Saxena (2005)
Ghee & cocoanut	*C. tetanii*	Hlady *et al*. (1992; Bennett *et al*. (1997)
Teas & tea flavonoids	*C. perfringens*	Friedman, 2007
Korean dung beetle	*C. difficile*	Kang *et al*. (2011)
Clematis hirsute	*C. chauvoei*	*Yineger et al.* (2007)
Rumex nepalensis	*C. chauvoei*	"
Leonotis ocymifolia	*Anthrax bacillus*	"
Cucumis ficifolius	*C. chauvoei*	"
Nigella sativa L.	*C.chauvoei*	"
Discopodium eremanthum Chiov.	*C. chauvoei*	"
Sonchus bipontini	*C. chauvoei*	"
Crepis rueppellii	*C. chauvoei*	"
Nicotiana tabaccum L.	*C. chauvoei*	"
Ruta chalepensis L	"	"
Sida schimperiana.	"	"
Sonchus bipontini	"	"
Cymbopogon citrates	"	"
Alchemilla abyssinica	"	"
Salvia nilotica	"	"
Vernonia myrantha	"	"
Senecio fresenii	"	"
Curcumin (from *Curcuma longa*) docked with α-toxin of *S. aureus*	*C. perfringens* δ- toxin	Skariyachan *et al*. (2011)
Tea phenolics	*C. difficile*	Lee *et al*. (2006)
Thearubigin	*C. botulinum*	Satoh *et al*. (2001)

It has been reported that tea catechins strongly inhibit the growth of *C. perfringens in vitro* (Hara *et al*., 1989; Ahn *et al*., 1991; Hara *et al*., 1995). Green tea catechins also reduced the heat-resistance characteristics of *C. hermoaceticum*, which proliferate in vending machines, causing sour spoilage in milk and other drinks (Sakanaka *et al*., 2000). Some other unpublished data (cited by Friedman, 2007) also suggest that concentrated green tea extracts inhibit sporulation and growth of *C. perfringens* in ground meat and turkey products during chilling. These observations suggest that the use of polyphenols or other natural antimicrobials may result in reduction of temperatures used in thermal processing of foods.

Coprisin, an antibacterial peptide has been isolated from *Copris tripartitus*, a Korean dung beetle, and a nine-amino-acid peptide identified in its α-helical region (Hwang *et al*., 2009). Kang *et al.* (2011) investigated the clinical significance of treatment with a coprisin analogue (a disulfide dimer of the nine peptides) on inflammation and mucosal damage in a mouse model of acute gut inflammation, established by administration of antibiotics followed by *C. difficile* infection. Coprisin treatment

significantly ameliorated decreases in body weight, improved survival rate, decreased mucosal damage and pro-inflammatory cytokine production. In contrast, the coprisin analogue had no apparent antibiotic activity against commensal bacteria, including *Lactobacillus* and *Bifidobacterium* spp, which are known to inhibit the colonization of the gut by *C. difficile*. The exposure of *C. difficile* to the coprisin analogue caused a marked increase in nuclear propidium iodide (PI) staining, indicating membrane damage. The staining levels were similar to those seen with bacteria treated with a positive control for membrane disruption (EDTA). In contrast, coprisin analogue treatment did not trigger increases in the nuclear PI staining of *Bifidobacterium thermophilum*. This observation suggests that the antibiotic activity of the coprisin analogue may occur through specific membrane disruption of *C. difficile*. Thus, these results indicate that the coprisin analogue may prove useful as a therapeutic agent for *C. difficile* -associated inflammatory diarrhoea and pseudo-membranous colitis.

Chinese traditional medicine has a long history which dates back thousands of years ago. To date, Chinese people still believe in traditional medicine and herbal medications are very popular in Chinese societies around the world. The latest encyclopedia of Chinese traditional medicine lists over 5500 natural sources (82.8% of which are plants) that form the basis for the 100,000–500,000 prescriptions of Chinese traditional medicine (Tang and Eisenbrand, 1992; Zhu and Woerdenbag, 1995). However, Chinese traditional medicine is unacceptable to Western society partly because the bases of Chinese traditional medicine like the yin-yang theory and five element theory (Cheng, 2000) are difficult to understand. Chinese herbal medicine has shown that the fruit and bark of plants belonging to the family Melia could be used as digestive tract-parasiticide and agricultural insecticide. Toosendanin (TSN, C30H38O11, FW=574), a triterpenoid derivative, was extracted from the bark of *Melia toosendan* Sieb. et Zucc. by Chinese scientists in 1950's and used as an ascarifuge in China instead of imported sendanin. Studies have demonstrated that TSN possesses special biological actions and perhaps considerable pharmacological values in scientific research, clinical medicine and agriculture. The first is that by interfering with neurotransmitter release by causing an initial facilitation, TSN eventually blocks synaptic transmission at both the neuromuscular junction and central synapses. The action might result from TSN-induced Ca^{2+}-sensitivity change and final elimination of transmitter release machinery. The second is that despite sharing many similar actions with botulinum neurotoxin (BoNT) on blocking neuromuscular transmission, TSN has a markedly anti-botulismic action *in vivo* and *in vitro*: TSN-treatment is reported to prevent death in mice and monkeys with botulism. TSN-incubation *in vitro* or TSN-injection *in vivo* endows neuromuscular junction with a high tolerance to BoNT. Studies suggest that the anti-botulismic action is achieved by preventing BoNT from approaching its enzymatic substrate, SNARE protein (Shi and Li, 2007).

Other studies on the role of TSN as anti-botulism natural product have been reported (Nakai *et al.*, 2009). Experiments carried out on phrenic nerve-diaphragm preparations of rats showed that the neuromuscular blocking action of TSN was similar to that of botulinum neurotoxin (BoNT) in many respects (Shi *et al*., 1980, 1981a,b, 1982). For example, they were both concentration- and temperature dependent; and the temperature coefficients were both high. Also, their dosage-response relationships were similar and the blocking effects were both not only irreversible but also related to the nerve activity and concentration of Ca^{2+}. There was always a delay between drug application and transmission block. Even if the drugs were washed out during the latent period, the blockade still occurred, indicating that both TSN and BoNT bound to their binding sites very fast. Similar to BoNT, TSN eventually blocked neurotransmitter release by abolishing Ca^{2+}-sensitivity of the transmitter release machinery. Moreover, some drugs, like guanidine and 4-aminopyridine, which facilitates neurotransmitter release and antagonize botulism, also have anti-TSN effect (Shi *et al*., 1981a). Except for sharing these similarities, the action of TSN is different from that of BoNT in some respects. For example, TSN changed excitability and fine structure of nerve endings and affected neurotransmitter release at central synapses as it did at the neuromuscular junction (Huang *et al.*, 1996; Shi and Chen, 1999; Chen *et al.*, 1999; Xu *et al*., 2004). The most prominent difference was that TSN always facilitated neurotransmitter release before blocking it. During the facilitatory phase, both the transmitter release (either spontaneous or evoked), and the Ca^{2+}- sensitivity of transmitter release machinery were enhanced (Shi *et al.*, 1981a, 1982; Xu *et al*., 2004). The similarities between the actions of TSN and BoNT suggest that TSN and BoNT may act at an adjacent site by a similar approach. However, analysis of their differences may give us a clue with which to reveal the anti-botulismic mechanism of TSN.

C. chauvoei, which is a very important veterinary pathogen is known to produce sialidase (neuraminidase), an enzyme reported to facilitate its spread in host tissues (Useh *et al*., 2004a; Useh *et al.*, 2006c). Many herbal remedies have been reported to ameliorate blackleg, caused by *C. chauvoei*, but the rational basis for this has yet to be demonstrated in the literature (Yineger *et al*., 2007). The Fulani pastoralists of rural Nigeria, who own livestock resources in the country, prefer the use of herbal remedies to treat animal diseases, including blackleg, because they are more natural, cheaper and safer (Jagun *et al.*, 1996; Gammaniel, 2000). *Tamarindus indicus* and *Combretum fragrans* are two common herbal remedies used recurrently by the nomads of rural Nigeria to treat blackleg (Abdu *et al.*, 2000). A study by some Nigerian authors to investigate the possible role of the herbs on blackleg showed that methanolic extracts of the stem barks of these plants inhibited sialidase (neuraminidase) activity *in vitro* in a dose-dependent manner. The authors therefore suspected that neuraminidase inhibition, leading to inability of the bacteria to spread in tissues, could be a major mechanism by which the herbs ameliorated the disease. The results revealed a target of action by the medicinal plants, thus corroborating their use in traditional medicine practices in Nigeria (Useh *et al*.,

2004b). Sialidases (neuraminidases) have been implicated in the pathologies of several diseases, including cell invasion in Chaga's disease, anaemia in trypanosomosis and viral invasion in Newcastle disease. The foregoing, according to the authors, makes the sourcing of neuraminidase inhibitors mandatory for treatment and amelioration of clinical symptoms related to the physiological activity of the enzyme. In most cases, the inhibitors are synthetic and indeed costly, e.g. Zanamivir used in the treatment of influenza virus infection. Moreover, some of the synthetic inhibitors become less effective on account of mutations, which are rampant in sialidases. This makes recourse to plants as source of neuraminidase inhibitors an appealing alternative, because their active compounds are synthesized in direct response to bacterial and viral invasion. In the said study, the two medicinal plants were tested, because of their reported role in ameliorating blackleg in traditional veterinary practice. These authors concluded that the active principles of these medicinal plants should be further characterized, to exploit the findings in drug development.

8. Conclusion

The future use of natural products in the clinical management of clostridial infections, although laced with a lot of potentials, is a puzzle that research should be intensified to resolve, considering the economic importance, with regards to the pathogenic, industrial and potential therapeutic uses of members of this genus. The emergence of drug resistant strains, with enhanced pathogenicity is deadly and requires concerted efforts to pull through this quagmire. Medical and veterinary research should be re-jigged to evolve active principles that are not so easily rendered inconsequential by the surge in drug resistant strains. Future research should focus on the possibility of targeting the critical virulence factors of clostridial agents using active principles from herbs, which have proved to be useful and potential therapeutic agents over time.

Acknowledgements

A United States Senior Fulbright Research Award to NM Useh at the Department of Population Medicine and Diagnostic Sciences, College of Veterinary Medicine, Cornell University, Ithaca, New York, where the manuscript was prepared is gratefully acknowledged. NM Useh also expresses appreciation to German Research Foundation (DFG), Bonn, Germany, The Academy of Sciences for the developing world (TWAS), Trieste, Italy and Deutscher Akademischer Austausch Dienst (DAAD), Bonn, Germany for generous financial support via clostridial research grants at various times.

References

Abdu PA, Jagun AG, Gefu JO, Mohammed AK, Alawa CBI and Omokanye AT. 2000. Ethnoveterinary practices, research and development. In: Proceedings of National Animal Production Research Institute (NAPRI), Ahmadu Bello University Zaria, Nigeria, 163.

Adams LG. 1998. Animal health issues in South Texas cattle. Workshop on beef cattle production systems and natural resources conservation in semi-arid lands of South Texas and Northern Mexico held at Universidad Auto'noma dr Tamaulipas, Cd. Victoria, Tamaulipas, Mexico, 26–27. February 1998.

Ahn Y- J, Kawamura T, Kim M, Yamamoto T and Mitsuoka T. 1991. Tea polyphenols: selective growth inhibitors of *Clostridium* spp. *Agric Biol Chem.*, **55**: 1425-1426.

Ahn Y-J, Lee C -O , Kweon J-H , Ahn V and Park J-H. 1998. Growth-inhibitory effects of Galla Rhois-derived tannins on intestinal bacteria. *J Appl Microbiol*., **84**: 439-443.

Alam SI, Bansod S and Singh L. 2008. Immunization against *Clostridium perfringens* cells elicits protection against *Clostridium tetani* in mouse model: identification of cross-reactive proteins using proteomic methodologies. *BMC Microbiol*., **8**: 194.

Al-Barrak A, Embil J, Dyck B, Olekson K, Nicoll D, Alfa M and Kabani A. 1999. An outbreak of toxin A negative, toxin B positive *Clostridium difficile*-associated diarrhoea in a Canadian tertiary-care hospital. *Canadian Comm Dis Rep*., **25**: 65-69.

Aldape M J, Bryant A E and Stevens D L. 2006. *Clostridium sordellii* infection: epidemiology, clinical findings, and current perspectives on diagnosis and treatment. *Clin Infec Dis*., **43**: 1436-1446.

Al- Saif N and Brazier JS. 1996. The distribution of *Clostridium difficile* in the environment of South Wales. *J Med Microbiol*., **45**: 133-137.

Amimoto K, Noro T, Oishi E and Shimizu M. 2007. A novel toxin homologous to large clostridial cytotoxins found in culture supernatant of *Clostridium perfringens* type C. *Microbiol*., **153**: 1198-1206.

Armstrong H L and MacNamee J K. 1950. Blackleg in deer. *J Amer Vet Med Assoc*., **117**: 212-214.

Arnon, S S, Schechter R, Inglesby T V, Henderson D A, Bartlett J G, Ascher M S, Eitzen E, Fine A D, Hauer J, Layton M, Lillibridge S, Osterholm M T, O'Toole T, Parker G, Perl T M, Russell P K, DSwerdlow D L, Tonat K and Working Group on Civilian Biodefense. 2001. Botulinum toxin as a biological weapon. *J Amer Med Assoc*., **285**: 1059-1070.

Arroyo L G, Kruth S A, Willey B M, Staempfli H R, Low D E and Weese J S. 2005. PCR ribotyping of *Clostridium difficile* isolates originating from human and animal sources. *J Med Microbiol*., **54**: 163-166.

Bagadi H O. 1977. The production and counting of spores of *Clostridium chauvoei*. *Appl Environ Microbiol*., **33**:1287-1288.

Bakri M. 2009. *Clostridium difficile* in ready to eat salads, Scotland. *Emerg Infect Dis*., **15**: 817-818.

Bandelj P, Trilar T, Racnik J, Zadravec M , Pirs T, Avbersek J, Micunovic J , Ocepek M and Vengust M. 2011. Zero prevalence of *Clostridium difficile* in wild passerine birds in Europe. *FEMS Microbiol Lett*., **321**, 183-185.

Bankar S B, Survase S A, Singha R S and Granström T. 2011. Continuous two stage acetone-butanol ethanol fermentation with integrated solvent removal using *Clostridium acetobutylicum* B 5313. *Biores Technol*. doi:10.1016/j.biortech.2011.12.

Bao G, Wang R, Zhu Y, Dong H, Mao S, Zhang Y, Chen Z, Li Y and Ma Y. 2011. Complete genome sequence of *Clostridium acetobutylicum* DSM 1731, a solvent-producing strain with multireplicon genome architecture. *J Bacteriol*., **193**: 5007-5008.

Barbe´ S S, van Mellaert L and Anne J. 2006. The use of clostridial spores for cancer treatment. *J Appl Microbiol*., **101**: 571-578.

Bartlett J G. 2007. Changing trends in bacterial infections: *Staphylococcus aureus*, bacterial pneumonia, *Clostridium difficile*. *Top HIV Med*., **15**: 94-98.

Bennett J, Macia J, Traverso H, Banoagha S, Malooly C and Boring J. 1997. Protective effects of topical antimicrobials against neonatal tetanus. *Int J Epidemiol*., **26**: 897-903.

Bossi P, Garin D, Guihot A, Gay F, Crance J-M, Debord T, Autran B and Bricaire F. 2006. Bioterrorism: management of major biological agents. *Cell Mol Life Sci*., **63**: 2196-2212.

Boyanovaa L, Kolarovb R, Gergovaa G and Mitov I. 2006. *In vitro* activity of Bulgarian propolis against 94 clinical isolates of anaerobic bacteria. *Anaerobe*, **12**: 173177.

Brusottia G, Cesaria I, Frassàa G, Grisoli P, Dacarroa C and Caccialanza G. 2011. Antimicrobial properties of stem bark extracts from *Phyllanthus muellerianus* (Kuntze) Excell. *J Ethnopharmacol*., **135**: 797-800.

Butler R. 1999. UNSCOM's Comprehensive Review. Available at: http://cns.miis.edu/iraq/ ucreport/.

Cato E P, George W L and Finegold S M. 1986. Genus *Clostridium*. In: Sneath PHA (Ed),. **Bergy's Manual of Systematic Bacteriology**. Volume 2. William and Wilkins, Baltimore, MD; pp. 1141-1182.

Centers for Disease Control and Prevention (CDC): Botulism in the United States, 1899-1996, handbook for epidemiologists, clinicians, and laboratory workers Atlanta, GA: CDC; 1998.

Chen E, Deng L, Liu Z, Zhu X, Chen X and Tang H. 2011. Management of gas gangrene in Wenchuan earthquake victims. *J Huazhong Uni Sci Technol Med Sci*., **31:** 83e7.

Chen W Y, Yin P B, Ye W L and Shi Y L. 1999. Toosendanin-induced change of dopamine level detected by microdialysis *in vivo* at rat striatum. *Chinese Sci Bull*., **44**: 1106-1110.

Cheng J T. 2000. Review: drug therapy in Chinese traditional medicine. *J Clin Pharmacol*., 40: 445-450.

Cottral G E. 1978. **Clostridia. Manual of Standard Methods for Veterinary Microbiology**. 1st ed. Cornell University Press, Ithaca, NY; p. 510–25.

Date K, Fagan R, Crossland S, Maceachern D, Pyper B, Bokanyi R, Houze Y, Andress E and Tauxe R. 2011. Three outbreaks of foodborne botulism caused by unsafe home canning of vegetables-Ohio and Washington, 2008 and 2009. *J Food Prot.*, **74(12)**: 2090-2096.

Desai R and Papoutsakis E. 1999. Antisense RNA strategies for metabolic engineering of *Clostridium acetobutylicum*. *Appl Environ Microbiol*., **65**: 936-945.

Dong H, Tao W, Dai Z, Yang L, Gong F, Zhang Y and Li Y. 2011. **Biobutanol. Advances in Biochemical Engineering/Biotechnology** DOI: 10.1007/10_2011_128. Springer-Verlag Berlin Heidelberg 2011.

Dong H, Zhang Y, Dai Z and Li Y. 2010. Engineering *Clostridium* strain to accept unmethylated *DNA*. *PLoS One*, **5**: e9038.

Eubanks L M, Dickerson T J and Janda K D. 2007. Technological advancements for the detection of and protection against biological and chemical warfare agents. *Chem Soc Rev*., **36**: 458-470.

Ezeji T, Milne C, Price N D and Blaschek H P. 2010. Achievements and perspectives to overcome the poor solvent resistance in acetone and butanol-producing microorganisms. *Appl Microbiol Biotechnol*., **85**: 1697-1712.

Ezeji T C, Qureshi N and Blaschek H P. 2007. Bioproduction of butanol from biomass: from genes to bioreactors. *Curr Opin Biotechnol*., **18**: 220-227.

Fasanella A, Galante D, Garofolo G and Jones M H. 2010. Anthrax undervalued zoonosis. *Vet Microbiol*., **140**: 318-331.

Floyd J G. 1994. Blackleg and other clostridial diseases in cattle. Extension Veterinarian, ANR-888, A Publication of the Alabama Cooperative Extension System; 3 pp.

Forgetta V, Oughton MT, Marquisi P, Brukner I, Blanchette R, Haub K, Magrini V, Mardis ER, Gerding DN, Loo VG, Miller MA, Mulvey MR, Rupnik M, Dascal A and Dewar K. 2011. Fourteen-genome comparison identifies DNA markers for severe-disease-associated strains of *Clostridium difficile*. *J Clin Microbiol*., **49**: 2230-2238.

Fox M E, Lemmon M J, Giaccia A J, Minton N P and Brown J M. 2000. Genetically modified *Clostridium* for gene therapy of tumors. *Meth Mol Med*., **35**: 413-418.

Franz D R, Jahrling P B, Friedlander A M, McClain D J, Hoover D L, Bryne W R, Pavlin J A, Christopher G W and Eitzen E M Jr. 1997. Clinical recognition and management of patients exposed to biological warfare agents. *J Amer Med Assoc*., **278**: 399-411.

Friedman M. 2007. Overview of antibacterial, antitoxin, antiviral, and antifungal activities of tea flavonoids and teas. *Mol Nutr Food Res.*, **51**: 116 -134.

Gadhi C A, Weber M, Mory F, Benharref A, Lion V, Jana V and Lozniewski A. 1999. Antibacterial activity of *Aristolochia paucineris* Pomel. *J Ethnopharmacol*., **67**: 87-92.

Gamboa-Coronado M M, Mau-Inchaustegui S and Rodríguez-Cavallini E. 2011. Molecular characterization and antimicrobial resistance of *Clostridium perfringens* isolates of different origins from Costa Rica. *Rev Biologie Tropica*, **59**:1479-85.

Gammaniel K S. 2000. Toxicity from medicinal plants and their products. *Nig J Prod Med.*, **4**: 4-8.

Garey K W, Salazar M, Shah D, Rodrigue R and DuPont H L. 2008. Rifamycin antibiotics for treatment of *Clostridium difficile*-associated diarrhoea. *Ann Pharmacother*., **42**: 827-835.

Garofolo G, Galante D, Serrecchia L, Buonavoglia D and Fasanella A. 2011. Development of a real time PCR taqman assay based on the TPI gene for simultaneous identification of *Clostridium chauvoei* and *Clostridium septicum*. *J Microbiol Meth*., **84**: 307-311.

Gilca R, Fortin E, Frenette C, Longtin Y and Gourdeau M. 2012. Seasonal variations in *Clostridium difficile* infections are associated with influenza and respiratory syncytial virus activity independently of antibiotic prescriptions: a time-series analysis in Quebec, Canada. *Antimicrobial Agents Chemother*.,**56(2):** 639-646

Gnerlich J L, Ritter J H, Kirby J P and Mazuski J E. 2011. Simultaneous necrotizing soft tissue infection and colonic necrosis caused by *Clostridium septicum*. *Surg Infect*., **12**: 501-506.

Goohuis A, Bakker D, Corver J and Debast S B. 2008. Emergence of *Clostridium difficile* infection due to a new hypervirulent strain, polymerase chain reaction Ribotype 078. *Clin Infect Dis*., **47**: 1162-1170.

Halm A, Wagner M, Köfer J and Hein I. 2010. Novel real-time PCR assay for simultaneous detection and differentiation of *Clostridium chauvoei* and *Clostridium septicum* in clostridial myonecrosis. *J Clin Microbiol*., **48**: 1093-1098.

Hara Y, Luo S J, Wickremasinghe R L and Yamanishi T. 1995. Use and benefits of tea. *Food Rev Int*., **11**: 527-542.

Hara Y, Watanabe M and Sakaguchi G. 1989. The fate of *Clostridium botulinum* spores inoculated into tea drinks. *Shokuhin Kogyo Gakkaishi*, **36**: 375-379.

Harris, L M, Welker N E and Papoutsakis E T. 2002. Northern, morphological, and fermentation analysis of *spo0A* inactivation and overexpression in *Clostridium acetobutylicum* ATCC 824. *J Bacteriol*., **184**: 3586-3597.

Harvey R B, Norman K N, Andrews K, Hume M E, Scanlan C M, Callaway T R, Anderson R C, and Nisbet D J. 2011a. *Clostridium difficile* in poultry and poultry meat. *Roger Food borne Pathogens and Dis*., **8**: 1321-1323.

Harvey R B, Norman K N, Andrews K, Norby B, Hume M E, Scanlan C M, Hardin M D and Scott H M. 2011b. *Clostridium difficile* in retail meat and processing plants in Texas. *J Vet Diag Invest*., **23**: 807-811.

Hatheway C L. 1990. Toxigenic clostridia. *Clin Microbiol Rev*., **3**: 66-98.

Heap J T, Pennington O J, Cartman S T, Carter G P and Minton N P. 2007. The ClosTron: a universal gene knock-out system for the genus *Clostridium. J Microbiol Meth*., **70**: 452-464.

Heap J T and Minton N P: Methods, 2009. International patent WO 2009/ 101400.

Heuermann D, Roggentin P, Kleinneidam R G and Schauer R. 1991. Purification and characterization of a sialidase from *Clostridium chauvoei* NC08596. *Glycoconj J*., **8**: 95-101.

Hlady W G, Bennette J V, Samadi A R, Begum J, Hafez A, Tarafdar AI and Boring J R. 1992. Neonatal tetanus in rural Bangladesh: risk factors and toxoid efficacy. *Amer J Public Hlth*., **82**: 1365-1369.

Holderman L V, Cato E P and More W E C. 1977. **Anaerobe Laboratory Manual**. 4th ed. Anaerobe Laboratory Virginia Polytechnic Institute and State University, Blacksburg, VA; pp. 1-156.

Hopman N E, Keessen E C, Harmanus C, Sanders I M, vanLeengoed L A, Kuijper E J and Lipman L J. 2011. Acquisition of *Clostridium difficile* by piglets. *Vet Microbiol*., **149(1-2):** 186-192.

Huang H Y, Zhou C W and Shi Y L. 1996. Toosendanin facilitates [3H] noradrenaline release from rat hippocampal slices. *Nat Toxins*, **4**: 92-95.

Humeau Y, Doussau F, Grant N and Poulain B. 2000. How botulinum and tetanus neurotoxins block neurotransmitter release. *Biochimie*., **82**: 427-446.

Hwang J-S, Lee J, Kim Y J, Bang H S, Yun E Y, Kim S R, Suh H J, Kang B R, Nam S H, Jeon J P, Kim I and Lee D G. 2009. Isolation and characterization of a defensin-like peptide (corprisin) from the dung beetle *Corpris tripartitus*. *Int J Peptides* doi:10.1155/2009/136284.

Jagun A G, Abdu P A, Mohammed A K, Alawa C B I, Omokanye A K, 1996. Survey of ethnoveterinary practices in Nigeria. Technical progress report on the survey conducted on ethnoveterinary practices in Nigeria, sponsored by International Development Research Centre (IDRC), Canada. pp 77-79.

Jhung M A, Thompson A D, Killgore G E, Zukowski W E, Songer G, Warny M, Johnson S, Gerding D N, McDonald L C and Limbago B M. 2008. Toxinotype V *Clostridium difficile* in humans and food animals. *Emerg Infect Dis*., **14**: 1039-1045.

Jia K, Zhu Y, Zhang Y and Li Y. 2011. Group II intron-anchored gene deletion in *Clostridium*. *Plos One*, **6**: e16693.

Johnson E A and Bradshaw M. 2001. *Clostridium botulinum* and its neurotoxins: a metabolic and cellular perspective. *Toxicon*, **39**: 1703-1722.

Johnson S and Gerding D N. 1998. *Clostridium difficile*-associated diarrhoea. *Clin Infect Dis*., **26**: 1027-1034.

Johnson S, Schriever C, Galang M, Kelly C P and Gerding D N. 2007. Interruption of recurrent *Clostridium difficile*-associated diarrhoea episodes by serial therapy with vancomycin and rifaximin. *Clin Infect Dis*., **44**: 846-848.

Jones D T and Kreis S. 1995. Origins and relationships of industrial solvent-producing clostridial strains. *FEMS Microbiol Rev*., **17**: 223-232.

Jones S W, Tracy B P, Gaida S M and Papoutsakis E T. 2011. Inactivation of δ^G F in *Clostridium acetobutylicum* ATCC 824 blocks sporulation prior to asymmetric division and abolishes δ^E and δ^G protein expression but does not block solvent formation. *J Bacteriol*., **193**: 2429-2440.

Kang J K, Hwang J S, Nam H J, Ahn K J, Seok H, Kim S-K, Yun E Y, Pothoulakis C, Lamont J T and Kim V. 2011. Mechanisms of action: physiological effects of the insect peptide coprisin prevents *Clostridium difficile*-mediated acute inflammation and mucosal damage through selective antimicrobial activity. *Antimicrobial Agents Chemother*., **55**: 4850-4857.

Keel K, Brazier J, Post K, Weese S and Songer, J. 2007. Prevalence of PCR ribotypes among *Clostridium difficile* isolates from pigs, calves, and other species. *J Clin Microbiol*., **45**: 1963-1964.

Keessen E C, Donswijk C J, Hol S P, Hermanus C, Kuijper E J and Lipman L J A. 2011. Aerial dissemination of *Clostridium difficile* on a pig farm and its environment. *Environ Res*., **111**: 1027-1032.

Keessen E C, Leengoed L A, Bakker D, vandenBrink K M, Kuijper E J and Lipman L J. 2010. Prevalence of *Clostridium difficile* in swine thought to have *Clostridium difficile* infections (CDI) in eleven swine operations in the Netherlands. *Tijdschr Diergeneeskd* **135**:134–137.

Keyburn AL, Boyce JD, Vaz P, Bannam TL, Ford ME, Parker D, Di Rubbo A, Rood J I and Moore R J. 2008. NetB, a new toxin that is associated with avian necrotic enteritis caused by *Clostridium perfringens*. *PLoS Pathog*., **4**: 26.

Kiel N, Ho V and Natalie A P. 2011. A case of gas gangrene in an immunosuppressed Crohn's patient. *World Gastroenterol*., **17**: 3856-3858.

Köpke M, Mihalcea C, Liew F, Tizard JH, Ali M S, Conolly J J, Al-Sinawi B and Simpson S D. 2011. 2,3-Butanediol Production by Acetogenic bacteria, an alternative route to chemical synthesis, using Industrial waste gas. *Appl Environ Microbiol*., **77**: 5467-5475.

Koene M G J, Mevius D J, Wagenaar A, Harmanus C, Hensgens M P M, Meetsma A M, Putirulan F F, van Bergen M A P and Kuijper E J. 2012. *Clostridium difficile* in Dutch animals: their presence, characteristics and similarities with human isolates. *Clin Microbiol Infect*.,**18**: 778-784.

Kuehne S A, Heap J T, Cooksley C M, Cartman S T and Minton N P. 2011. *Strain Engineering: Methods and Protocols*, James A. Williams (Ed), **Methods in Molecular Biology**, vol. 765, DOI 10.1007/978-1-61779-197-0-23, Springer Science and Business Media,

Kuijper E J, Coignard B and Tull P. 2006. Emergence of *Clostridium difficile*-associated disease in North America and Europe. *Clin Microbiol Infect*., **12**: 2-18.

Kuske C R. 2006. Current and emerging technologies for the study of bacteria in the outdoor air. *Curr Opin Biotechnol*., **17**: 291-296.

Kyne L, Hamel M B, Polavaram R and Kelly C P. 2002. Health care costs and mortality associated with nosocomial diarrhoea due to *Clostridium difficile*. *Clin Infect Dis*., **34**: 346-353.

Kyne L, Warny M, Qamar A and Kelly C P. 2001. Association between antibody response to toxin A and protection against recurrent *Clostridium difficile* diarrhoea. *Lancet*, **357**: 189-193.

Lamanna C. 1959. The most poisonous poison. *Science*, **130**: 763-772.

Lange M, Neubauer H and Seyboldt C. 2010. Development and validation of a multiplex real-time PCR for detection of *Clostridium chauvoei* and *Clostridium septicum*. *Mol Cell Probes*., **24**: 204-210.

Lecour H, Ramos H, Almeida B and Barbosa R. 1998. Food borne botulism. A review of 13 outbreaks. *Arch Int Med*., **148**: 578-580.

Lee H C, Jemmer A M, Low C S and Lee Y K. 2006. Effects of tea phenolics and their aromatic faecal bacterial metabolites on intestinal microbiota. *Res Microbiol*., **157**: 876-884.

Leffler D A and Lamont J T. 2009. Treatment of *Clostridium difficile*-associated disease. *Gastroenterol*., **136**: 1899-1912.

Legaria M C, Lumelsky G and Rosetti S, 2003. *Clostridium difficile*-associated diarrhoea from a general hospital in Argentina. *Anaerobe*, **9**: 113-116.

Lütke-Eversloh T and Bahl H. 2011. Metabolic engineering of *Clostridium acetobutylicum*: recent advances to improve butanol production. *Curr Opin Biotechnol*., **22**: 634-647.

MacLennan J D. 1943a. Anaerobic infections of war wounds in the Middle East. *Lancet*, **242**: 94e9.

MacLennan J D. 1943b. Anaerobic infections of war wounds in the Middle East. *Lancet*, **242**: 123e6.

MacLennan J D. 1943c. Anaerobic infections of war wounds in the Middle East. *Lancet*, **242**: 63e6.

MacLennan J D. 1962. The histotoxic clostridial infections of man. *Bacteriol Rev*., **26**: 177e276.

MacLennan J D and MacFarlane R G. 1945. Toxin and antitoxin studies of gas gangrene in man. *Lancet*, **246**: 301e5.

Mangold T and Goldberg J. 1999. **Plague Wars: A True Story of Biological Warfare**. London: Macmillan.

Mao S, Luo Y, Bao G, Zhang Y, Li Y and Ma Y. 2011. Comparative analysis on the membrane proteome of *Clostridium acetobutylicum* wild type strain and its butanol-tolerant mutant. *Mol BioSystems*, **7**: 1660-1677.

Marks J D. 2004. Medical aspects of biologic toxins. *Anesthesiol Clin North Amer*., **22**: 509- 532.

Masuyer G, Stancombe P, Chaddock J A and Acharya K R. 2011. Structures of engineered *Clostridium botulinum* neurotoxin derivatives. *Acta Crystallographica*, **F67**, 1466-1472.

McClane BA. 2007. *Clostridium perfringens*. In: Doyle MP, Beuchat LR, (Eds),**Food Microbiology: Fundamentals and Frontiers,** 3rd ed. Washington, D.C.: ASM Press; 2007. pp. 423-444.

McDonald LC. 2005. *Clostridium difficile*: responding to a new threat from an old enemy. *Infect Control Hosp Epidemiol*., **26**: 672-675.

McFarland LV. 2006. Meta-analysis of probiotics for the prevention of antibiotic associated diarrhoea and the treatment of *Clostridium difficile* disease. *Amer J Gastroenterol*., **101**: 812-822.

Merchant I A and Barner R D. 1964. **An Outline of the Infectious Diseases of Domestic Animals**. Iowa State University Press, Ames, IA, pp. 22-26.

Metcalf D S, Costa M C, Dew W M V and Weese J S. 2010. *Clostridium difficile* in vegetables, Canada. *Lett Appl Microbiol*., **51**: 600-602.

Mhoma J R L, Rwehabula P, Kundy D J and Msangi N F. 1995. Assessment of blackquarter vaccine (AWASA strain) by mass vaccination of cattle and sheep in Tanzania. *Bull Anim Hlth Prod Afr*., **43**: 227-30.

Middlebrook J and Franz D. 1997. Botulinum toxins. In: Zajtchuk R. (ed.) ,**Textbook of Military Medicine: Medical Aspects of Chemical and Biological Warfare**, US Department of the Army, Surgeon General, and the Borden Institute, Washington, DC. pp. 643–654

Minton N P. 2003. Clostridia in cancer therapy. *Nature Rev Microbiol*., **1**: 237-242.

Mulvey M R, Boyd D A, Gravel D, Hutchinson J, Kelly S, McGeer A, Moore D, Simor A, Suh K N, Taylor G, Weese J S and Miller M. 2010. Hypervirulent *Clostridium difficile* strains in hospitalized patients, Canada. *Emerg Infect Dis*., **16**: 678-681.

Nagano N, Isomine S, Kato H, Sasaki Y, Takahashi M, Sakaida K, Nagano Y and Arakawa Y. 2008. Human fulminant gas gangrene caused by *Clostridium chauvoei*. *J Clin Microbiol*., **46**: 1545-1547.

Nakai Y, Tepp W H, Dickerson T J, Johnson E A and Janda K D. 2009. Function-oriented synthesis applied to the anti-botulinum natural product toosendanin. *Bioorg Med Chem*., **17**: 1152-1157.

Nakayama S, Kiyoshi K, Kadokura T and Nakazato A. 2011. Butanol Production from crystalline cellulose by cocultured *Clostridium thermocellum* and *Clostridium saccharoperbutylacetonicum* N1-4. *Appl Environ Microbiol*., **77**: 6470-6475.

Nwokediuko S, Bababode B, Chinedu OU and Ozoh G. 2002. Peculiarities of chronic diarrhoea in Enugu, Southeastern Nigeria. *J Hlth Sci*., **48**: 435-440.

Otte B, Grunwaldt E, Mahmoud O and Jennewein S. 2009. Genome shuffling in *Clostridium diolis* DSM 15410 for improved 1,3-propanediol production. *Appl Environ Microbiol*., **75**: 7610-7616.

Papoutsakis E T. 2008. Engineering solventogenic clostridia. *Curr Opin Biotechol*., **19**: 420-429.

Paredes-Sabja D and Sarker M R. 2012. Interactions between *Clostridium perfringens* spores and Raw 264.7 macrophages interactions between *Clostridium perfringens* spores and Raw 264.7 macrophages. *Anaerobe, **18(1): 148-156.***

Paredes-Sabja D and Sarker M R. 2009. *Clostridium perfringens* sporulation and its relevance to pathogenesis. *Future Microbiol*., **4**: 519-525.

Radostits O M, Gay C C, Blood D C and Hinchcliff KW. 2000. **Veterinary Medicine. A Textbook of the Diseases of Cattle, Sheep, Pigs, Goats and Horses.** 9th ed. W.B Saunders Company Ltd, London, pp. 760-763.

Ramarao D and Rao BU. 1990. Studies on the incidence of blackquarter in Karnataka during 1979-1985. *Indian Vet J*., **67**:795-801.

Rodloff A C and Krüger M. 2012. Chronic *Clostridium botulinum* infections in farmers. *Anaerobe*, **18(2):** 226-228.

Rotz L D, Khan A S, Lillibridge S R, Ostroff S M and Hughes J M. 2002. Public health assessment of potential biological terrorism agents. *J Amer Med Assoc*., **8**: 225-230.

Rupnik M, Wilcox M H and Gerding D N. 2009. *Clostridium difficile* infection: new developments in epidemiology and pathogenesis. *Natures Rev Microbiol*., **7**: 526-36.

Rupnik M. 2007. Is *Clostridium difficile*-associated infection a potentially zoonotic and food borne disease ? *Clin Microbiol Infect*., **13**: 457-459.

Sakanaka S, Juneja L R and Taniguchi M. 2000. Antimicrobial effects of green tea polyphenols on thermophilic spore-forming bacteria. *J Biosci Bioeng*., **90**: 81-85.

Satoh E, Ishii T, Shimizu Y, Sawamura S and Nishimura M. 2001. Black tea extract, thearubigin fraction, counteracts the effects of botulinum neurotoxins in mice. *Exper Biol Med*., **226**: 577-580.

Saxena S. 2005. *Glycyrrhiza glabra* medicine over the millennium. *Nat Prod Rad*., **4**: 358-367.

Schantz E J and Johnson E A. 1992. Properties and use of botulinum toxin and other microbial neurotoxins in medicine. *Microbiol Rev*., **56**: 80-99.

Seal D, Borriello SP, Barclay F, Welch A, Piper M and Bonnycastle M. 1987. Treatment of relapsing *Clostridium difficile* diarrhoea by administration of a non-toxigenic strain. *Eur J Clin Microbiol*., **6**: 51-53.

Shapiro R L, Hatheway C and Swerdlow D L. 1998. Botulism in the United States: a clinical and epidemiologic review. *Ann Int Med*., **129**: 221-228.

Shi Y L and Chen W Y. 1999. Effect of toosendanin on acetylcholine release from rat brain, a microdialysis study. *Brain Res*., **850**: 173-178.

Shi Y–L and Li M-F. 2007. Biological effects of toosendanin, a triterpenoid extracted from Chinese traditional medicine. *Prog Neurobiol*., **82**: 1-10.

Shi Y L, Wang W P and Xu K. 1981a. Electrophysiological analysis on the presynaptic blocking effects of toosendanin on neuromuscular transmission. *Acta Physiol Sin*., **33**: 259-265.

Shi Y L, Wang W P, Yan S Q and Xu K. 1982. Effects of calcium ions and nerve impulses on changes in miniature end-plate potential frequency produced by toosendanin. *Acta Physiol Sin*., **34**: 304-309.

Shi Y L, Wei N S, Yang Y Q and Wang Z X. 1980. Toosendanin-a presynaptic neuromuscular blocking agent. *Acta Physiol Sin*., **32**: 293-297.

Shi Y L, Yang Y Q, Wang W P and Xu K. 1981b. Influence of stimulus frequency, temperature and Ca^{2+} on onset of toosendanin paralysis of isolated neuromuscular preparations of rats. *Acta Physiol Sin*., **33**: 141-146.

Simango C and Mwakuruddwa S. 2008. *Clostridium difficile* in broiler chickens sold at market places in Zimbabwe and their antimicrobial susceptibility. *Int J Food Microbiol*., **10**: 268-270.

Skariyachan S, Mahajanakatti A B, Sharma N and Sevanan M. 2011. Selection of herbal therapeutics against deltatoxin mediated clostridial infections. *Bioinfo*., **6**: 375-379.

Songer J G and Uzal F A. 2005. Clostridial enteric infections in pigs. *J Vet Diag Investig*., **17**: 528-536.

Songer J G, Trinh H T, Killgore G E, Thompson A D, McDonald L C and Limbago B M. 2009. *Clostridium difficile* in retail meat products, USA, 2007. *Emerg Infect Dis*., **15**:819-821.

Sosa A. 2005. Antibiotic policies in developing countries. In: Gould IM, van der Meer J W M (Eds), **Antibiotic Policies: Theory and Practice**. New York: Kluwer Academic/Plenum, pp. 591-616.

Stabler R A, Gerding D N, Songer J G, Drudy D, Brazier J S, Trinh H T, Witney A A, Hinds J and Wren B W. 2006. Comparative phylogenomics of *Clostridium difficile* reveals clade specificity and microevolution of hypervirulent strains. *J Bacteriol*., **188**:7297-7305.

Stevens D L, Aldape M J and Bryant A E. 2012. Life-threatening clostridial infections. *Anaerobe* , **18(2):** 254-259.

Survase S A, Sklavounos E, Jurgens G, van Heiningen A and Granström T. 2011. Continuous acetone-butanol-ethanol fermentation using SO_2-ethanol-water spent liquor from spruce. *Biores Technol*., **102**: 10996-11002.

Tang W and Eisenbrand G. 1992. **Chinese Drugs of Plant Origin: Chemistry, Pharmacology, and Use in Traditional and Modern Medicine**. Springer, Heidelberg.

Titball R W. 2009. *Clostridium perfringens* vaccines. *Vaccine*, **27**: D44-D47.

Tomas C A, Beamish J and Papoutsakis E T. 2004. Transcriptional analysis of butanol stress and tolerance in *Clostridium acetobutylicum*. *J Bacteriol*., **186**: 2006-2018.

Troxel T R, Burke G L, Wallace W T, Keaton L W, McPeake S R, Smith D and Nicholson I. 1997. Clostridial vaccination efficacy on stimulating and maintaining an immune response in beef cows and calves. *J Anim Sci*., **75**:19-25.

Tummala S B, Welker N E and Papoutsakis E T. 2003. Design of antisense RNA constructs for downregulation of the acetone formation pathway of *Clostridium acetobutylicum*. *J Bacteriol*., **185**: 1923-1934.

Twine S M, Reid C W, Aubry A, McMullin D R, Fulton K M, Austin J and Logan S M. 2009. Motility and flagellar glycosylation in *Clostridium difficile*. *J Bacteriol*., **191**: 7050-7062.

Useh N M, Ajanusi J O, Esievo K A N and Nok A J. 2006c. Characterization of a sialidase (neuraminidase) from *Clostridium chauvoei* (Jakari strain). *Cell Biochem Funct*., **24**: 347-352.

Useh N M, Ibrahim N D G, Nok A J and Esievo K A N. 2006b. Relationship between outbreaks of blackleg of cattle and annual rainfall in Zaria, Nigeria. *Vet Rec*., **158**:100-101.

Useh N M, Nok A J, Ajanusi O J, Balogun E O, Oladele S B and Esievo K A N. 2004a. *In vitro* production of neuraminidase by *Clostridium chauvoei* (Jakari strain). *Vet Arh*., **74**: 289-298.

Useh N M, Nok A J, Ambali S F and Esievo K A N. 2004b. Inhibition of *Clostridium chauvoei* (Jakari strain) neuraminidase activity by methanolic extracts of the stem barks of *Tamarindus indicus* and *Combretum fragrans*. *J Enzyme Inhib Med Chem*., **19**: 339-42.

Useh N M, Nok A J and Esievo K A N. 2006a. Blackleg in ruminants. *Persp Agric Vet Sci Nutr Nat Res*., 1: 040.

Uzal F A, Paramidani M, Assis R, Morris W and Miyakawa M F. 2003. Outbreak of clostridial myositis in calves. *Vet Rec*., **152**: 134-136.

von Abercron S M M, Karlsson F, Wigh G T, Wierup M and Krovacek K. 2009. Low Occurrence of *Clostridium difficile* in retail ground meat in Sweden. *J Food Prot*., **72**: 1732-1734.

Warny M, Pepin J, Fang A, Killgore G, Thompson A, Brazier, Frost E and McDonald L C J. 2005. Toxin production by an emerging strain of *Clostridium difficile* associated with outbreaks of severe disease in North America and Europe. *Lancet*, **366**: 1079-84.

Weatherhead J E and Tweardy D J. 2012. Lethal human neutropenic entercolitis caused by *Clostridium chauvoei* in the United States: Tip of the iceberg? *J Infect*., **64**: 225-227. doi:10.1016/j.jinf.2011.09.004.

Weese J S, Finley R, Reid-Smith R R, Janecko N and Rousseau J. 2010. Evaluation of *Clostridium difficile* in dogs and the household environment. *Epidemiol Infect*., **138**: 1100-1104.

Weese J S. 2010. *Clostridium difficile* in food-innocent by stander or serious threat? *Clin Microbiol Infect*., **16**:3-10.

Weese J S, Staempfli H R, Prescott J F, Kruth S A, Greenwood S J and Weese H E. 2001a. The roles of *Clostridium difficile* and enterotoxigenic *Clostridium perfringens* in diarrhoea in dogs. *J Vet Int Med*., **15**: 374-378.

Weese J S, Weese H E, Bourdeau T L and Staempfli H R. 2001b. Suspected *Clostridium difficile* associated diarrhoea in two cats. *J Amer Vet Med Assoc*., **218**: 1436-1439.

Weingart O G, Schreiber T, Mascher C, Pauly D, Dorner M B, Berger T F H, Egger C, Gessler F, Loessner M J, Avondet M-A and Dorner B G. 2010. The case of botulinum toxin in milk: experimental data. *Appl Environ Microbiol*., **76**: 3293-3300.

Williams B M. 1977. Clostridal myositis in cattle: bacteriology and gross pathology. *Vet Rec*., **100**: 90-91.

Williams B M, Andrews A H. Bacterial conditions. 1992. In: Andrews AH, Lowrey RW, Boyd H, Eddy RG, editors. **Bovine Medicine, Diseases and Husbandry of Cattle**. Blackwell Scientific Publications, Oxford, pp. 551-557.

Woodford N and Livermore D M. 2009. Infections caused by Gram-positive bacteria: a review of the global challenge. *J Infect*., **59** Suppl 1: S4.

World Health Organization (WHO), 2002. WHO Traditional Medicine Strategy 2002–2005. World Health Organization, Geneva.

Xiu Z L and Zeng A P. 2008. Present state and perspectives of downstream processing of biologically produced 1,3-propanediol and 2,3-butanediol. *Appl Microbiol Biotechnol*., **78**: 917-926.

Xu T H, Shi Y L, Ren X M and Zhou J Y. 2004. Inhibition of glutamate release from rat cerebral synaptosomes by a prejunctional blocker toosendanin. *Chinese J Pharmacol Toxicol*., **18**: 321-326.

Yen H-W, Lin S-F and Yang I-K. 2012. Use of poly (ether-block-amide) in pervaporation coupling with a fermentor to enhance butanol production in the cultivation of *Clostridium acetobutylicum*. *J Biosci Bioeng*., **113(3):** 372-377.

Yineger H, Kelbessa E, Bekele T and Lulekal E. 2007. Ethnoveterinary medicinal plants at Bale Mountains National Park, Ethiopia. *J Ethnopharmacol*., **112**: 55-70.

Yu M, Zhang Y, Tang I-C and Yang S-T. 2011. Metabolic engineering of *Clostridium tyrobutyricum* for n-butanol production. *Metabolic Eng*., **13**: 373-382.

Zhang L, Leyn S A, Gu Y, Jiang W, Dmitry A and Yang R C. 2012. Ribulokinase and transcriptional regulation of arabinose metabolism in *Clostridium acetobutylicum*. *J Bacteriol*., **194(5):** 1055-1064.

Zhu Y P and Woerdenbag, H J. 1995. Traditional Chinese herbal medicine. Pharmacy World. *Science*, 17: 103-112.

JJBS

Jordan Journal of Biological Sciences

Volume 7, Number 2, June .2014
ISSN 1995-6673
Pages 95 - 100

A Survey of the Processing and Chemical Composition of Gariss Produced by Nomadic Camel Women Herders in AlGaderif State, Sudan

Eilaf S. K. Suliman and Ibtisam E. M. El Zubeir*

Department of Dairy Production, Faculty of Animal Production. University of Khartoum, Khartoum North, P. O. Box 32, Postal code 13314, Sudan

Received: November 2, 2013 Revised: December 27, 2013 Accepted: January 3, 2014

Abstract

Gariss is the most popular stable food for the camel herders who depend on Gariss for the sustainability of their livestock. The objective of the current study is to assess the traditional fermented camel milk (Gariss) prepared by nomadic camel woman herders in AlGadarif State in Butana. It is also meant to improve the quality of camel milk products through sharing knowledge. The samples (n= 19) were collected during rainy and dry seasons. The nomad's housekeepers were interviewed in the study area. The effect of different types of containers and the additives used during Gariss preparation and the compositional quality were all estimated. The survey reported the different types of spoilage and the variations in the shelf life of Gariss. When compared with the mean values of the Gariss samples collected during dry season, the result indicated that the values of the total solids and the pH of the samples collected during the rainy season were significantly higher ($p<0.05$), whereas the values of the fat, protein and ash were significantly lower ($p<0.05$). The container types had a significant ($p<0.05$) effect on the total solids, fat and ash content only. The Gariss prepared in Bukhsa showed the highest total solids content (13.15±0.54%) and that prepared in stainless steel showed the highest fat (4.65± 0.34%) content. However, when Gariss was prepared in plastic containers it showed the lowest pH value (3.59±0.16%), whereas samples from ''Siin'' (goat leather) was significantly lower ($p<0.05$) in the ash content (0.35± 0.09%). The present study concludes that the chemical composition of Gariss from the nomadic camel women herders is affected by seasons, types of additives and containers used. Hence more studies are needed to be done on the effect of the additive and containers on Gariss quality.

Keywords: Fermented Camel Milk, Gariss, Processing, Additives, Containers, Seasonal Movement, Nomadic Women, Sudan.

1. Introduction

Various fermented milk products, that are made of camel milk, include Gariss (Abdelgadir *et al.*, 1998; Hassan *et al.*, 2007 and Hassan *et al.*, 2008), yoghurt (Elayan *et al.*, 2008; Hashim *et al.*, 2008; El Zubeir *et al.*, 2012), fermented milk (Ashmaig *et al.*, 2009; El Zubeir and Ibrahium, 2009; Ahmed *et al.*, 2010) and cheese (El Zubeir and Jabreel, 2008). The fermented camel milk in Sudan (Gariss) is a semi-continuous fermentation process without the addition of any types of starter cultures; it is carried out in or outside the field prepared by shepherds when driving the camel for pastures in faraway places (Shori, 2012). Those herders depend on Gariss for several months as the sole source of various nutrients (Abdelgadir *et al.*, 1998).

Traditionally, fermented camel milk is allowed to ferment naturally without prior heat treatment and without addition of starter cultures (Abdelgadir *et al.*, 1998; Hassan *et al.*, 2008; Shori, 2012). Its final products have various names in different parts of the world. For example, in Sudan and Somalia, it is known as 'Gariss' (sour); however, in Sudan, it is also known as *hameedh* or *humadah*, which also means sour. It has substantial amounts of ethanol because of the acid alcoholic that is produced during milk fermentation (Dirar, 1993).

The method of Gariss preparation was described by various researchers (Dirar, 1993; Abdelgadir *et al.*, 1998; Elayan *et al.*, 2008; Hassan *et al.*, 2008; Ashmaig *et al.*, 2009; Ahmed *et al.*, 2010; El Zubeir and Ibrahium, 2009). Gariss is fermented in a large skin bag (locally named "Siin" which contains a large quantity of previously sour product, while in the absence of starter from previous lot, fermentation is initiated by adding, to the container, a few seeds of black cumin (*Nigellica sativa*) and one onion bulb (Dirar, 1993; Hassan *et al.*, 2008; and Ahmed *et al.*, 2010). Fermentation of Gariss takes place while the camels are on move and due to the inherent jerk in the camel's walk; the milk in the bags is gently shaken during fermentation (Mirghani, 1994).

* Corresponding author. e-mail: Ibtisammohamed@hotmail.com.

The chemical composition of Gariss was found to range of 2.8–5% fat and 10–11% total solids (Hassan *et al*., 2008; El Zubeir and Ibrahium, 2009), which were within the range of fresh camel milk: 1.8–5% of fat and 7.8–12% of total solids as stated by Shuiep *et al.* (2008). The range of pH for Gariss was 3.6–5.9 (Abdelgadir *et al.*, 2008; Hassan *et al.*, 2008; Ahmed *et al*., 2010) and the acidity as lactic acid ranged from 2.2–2.3% (Hassan *et al*., 2008).

The practice of camel herding is very well documented in Sudan, since some of the tribes of Sudan rely completely on herding and the pastoral is their life style (El Zubeir and Nour, 2006). Camels in Sudan are concentrated in two main regions; the Eastern (the Butana plains and the Red Sea hills) and the Western regions (Darfour and Kordofan). This study is conducted in order to get information about the processing techniques of Sudanese fermented camel milk prepared by nomadic women camel herders in Butana area. It was also aimed to improve the quality of camel milk products through sharing knowledge with nomadic women on the process of traditional fermentations process and how it can be controlled. Thus the present study was designed to evaluate the processing conditions of Gariss and to assess the effect on its compositional content.

2. Material and Methods

2.1. Area of Study and Target Groups

AlGedarif State, which is located in the eastern part of Sudan, is the area selected to perform this study. The camel herders chosen for this study belong to Elhlaween tribes who stay (settlement) in Butana plains during the rainy season (May to October), into the northern part of AlGedarif State and towards the southern part of the state from November to April to take the maximum advantage of the natural grazing and water resources (movement during the dry season). Nomadic livestock owners who used to find ample dry season resources (water and grazing) in the Atbra valley now traverse the area and take their animals across the border with Ethiopia, and, in most cases in the dry season, they buy the crop residues remaining from the irrigated schemes after the harvest.

2.2. Collection of Data

The nomad's housekeepers (n= 19) from the selected camel herding society were interviewed using the structural prepared questionnaire in order to assess the manufacture of local fermented camel milk (Gariss). The main parts of the questionnaire include camel milk products, traditional preserving methods, the containers used for processing, the methods of processing whether it is continuous or fed batch, the additives used and the methods of addition. Moreover, some questions about the shelf life and the defects and spoilage faced by the women herders were also included.

2.3. Collection of Samples

About 19 samples of Gariss (approximately 100 ml) were collected into a 250 ml sterile screw-capped bottle. The samples were collected during 24-36 hours every visits (n=3) and kept at 4° C until being brought to Khartoum in an ice bag. The pH of each sample was measured at the field and the chemical analysis was performed at the laboratory.

2.4. Chemical Analysis of the Samples

The pH was determined using pH meter (pH HANNA Instruments pH 211 Microprocessor pH Meter) according to Bradley *et al.* (1992). Titratable acidity was determined according to AOAC (1990a). The total solids content of the samples was determined according to the modified method of AOAC (1990b) and the ash content was determined according to AOAC (1990c). In addition, the fat content was determined by Gerber method as described by Bradley *et al.* (1992). Finally, the protein content was determined by Kjeldahl method according to AOAC (1990d).

2.5. Statistical Analysis

The data were analyzed using a completely randomized design. The significant differences between means were determined using Least Significant Different using statistix 8. The figures were plotted using Microsoft excel program.

3. Results

3.1. Gariss Processing Methods and Properties, Spoilage Occurrences and Shelf Life

This result indicated that 10.5% of the nomadic camel herders used Bukhsa (wooden Gourd) for preparing Gariss, 42.1% used plastic containers, and 42.1% used Siin and only 5.3% of the nomadic camel herders used stainless steel containers. On the other hand, the results of the survey clearly demonstrated that the nomadic camel herders preferred using plastic containers and Siin to prepare Gariss during the rainy season more than stainless steel. However, during the dry season they preferred using Bukhsa for preparing the Gariss (Figure 1). Figure 2 demonstrates that there were wide varieties of additives used for preparing Gariss; about 31.58% of households prepared Gariss without additives (plain), 21.05% of them used black cumin seeds (*Nigella sativa*) and onion (*Allium cepa*), 10.53% used ginger (*Zingiber officinale*) and black cumin seeds. The onion and fenugreek (*Trigonella foenum-graecum*) used by 15.78% of them, while 10.53% used onion, ginger, as well as fenugreek and grangal (*Alpinia galangal*). Moreover, the methods of adding these spices are varied. Some of them used the additives as powder in a piece of tied cloth and others add them directly without grinding.

The results of the survey indicate that different types of spoilage occurred (Figure 3); 26.3% of the household keepers observed a deterioration in Gariss as it became sour, and about 31.6% observed a formation of flakes/curd particles. Most of the nomadic households interviewed in the study area stated that camel milk is consumed either fresh or fermented, but some of them preferred it fresh (40%).

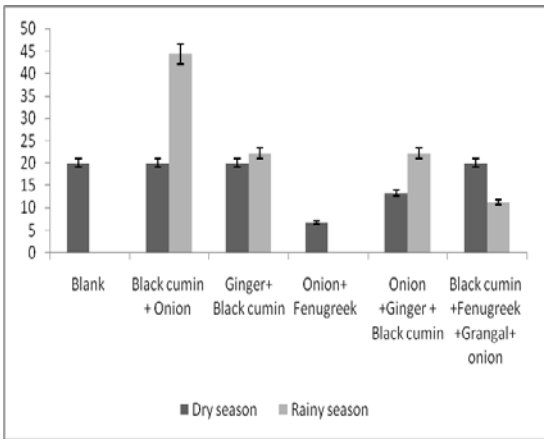

Figure 1. Percentage of different containers used in Gariss processing by nomadic camel women herders, AlGadarif State

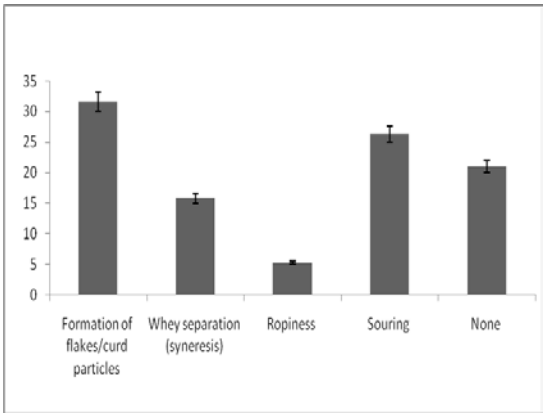

Figure 2. Percentage of different additives use in Gariss processing during the two seasons by nomadic camel women herders, AlGadarif State

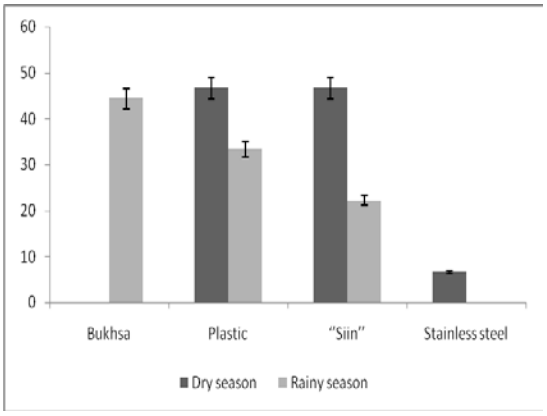

Figure 3. Percentage of different types of spoilage of Gariss faced by nomadic camel women herders, AlGadarif State

The ropiness defects represented about 5.3%, and 15.8% observed whey separation (syneresis), whereas, about 21% reported that no spoilage occurred. However the data in Figure 4 showed that 31.6% of the interviewed household keepers mentioned that the shelf life for the Gariss might extend up to 7 days, while 26.3% of them stated that it may extend up to 4 days. The shelf life proportions could be 5.3%, 21 %, and 15.8% for 24 hours, 48 hours and 3 days, respectively.

The most preferred camel milk is sour or fermented; this is suitable in the desert because of the high ambient temperature prevailing in the area which is coupled with the lack of cooling facilities that reduce the shelf life of milk.

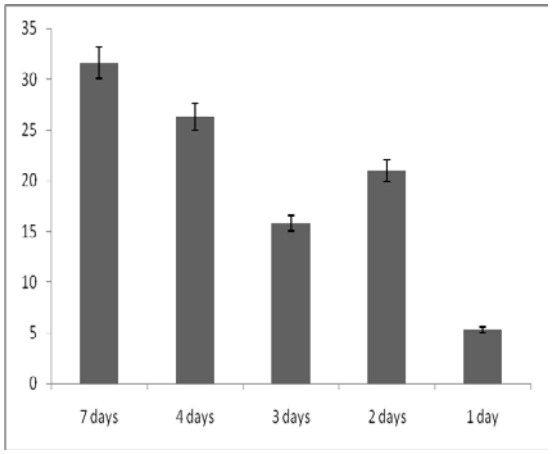

Figure 4. Percentage of different shelf lives of Gariss prepared by nomadic camel women herders, AlGadarif State

3.2. Chemical Composition of Gariss Samples Collected During Dry and Rainy Seasons of Nomadic Camel Herders, AlGadarif State

Table 1 shows that there are significant (p<0.05) differences of Gariss samples collected during dry compared to the rainy seasons concerning total solids (11.37%± 0.16 vs. 13.22%± 0.32), fat (3.73%± 0.11 vs. 3.06%± 0.22,), protein (4.88%± 0.14 vs. 3.97%± 0.27), ash (0.93%± 0.05 vs. 0.28%± 0.10), and pH (3.64± 0.1 vs. 4.52± 0.20). The highest total solids content (13.15± 0.54%) was found in Gariss prepared in Bukhsa compared to that prepared in plastic containers 11.88± 0.27%) followed by Gariss prepared in stainless steel containers (11.85± 0.76%) and ‘‘Siin’’ (11.28± 0.27%). The fat content of Gariss prepared using stainless steel container (4.65± 0.34%) was significantly higher (p<0.05) when compared to that prepared using ‘‘Siin’’ (3.71±0.12%), plastic containers (3.65± 0.12%) and Bukhsa (2.35± 0.24%) as shown in Table 2. The ash content of Gariss prepared in ‘‘Siin’’ (0.35± 0.09%) was significantly low (p<0.05) compared to that prepared using stainless steel (1.18±0.25%), plastic containers (0.76±0.09%) and Bukhsa (0.89±0.18%). However, the type of containers had an insignificant effect (p>0.05) on the acidity or the protein content of the Gariss. In regards to the type of containers, the lowest pH value (3.59± 0.16) was observed in Gariss prepared in plastic containers.

Table 3 shows that the additives types had a significant effect on the total solid contents. The total solids of Gariss prepared using mixtures of onion, black cumin, fenugreek and grangal (14.4%± 0.58) and Gariss with mixtures of onion, ginger and black cumin (13.15± 0.41%) were significantly higher (p<0.05) than the total solids of Gariss using other mixtures (10.85± 0.41% and 11.05 ±0.41%) or using no additives (11.39±0.23%). The lowest total solids content (10.85± 0.14%) was observed in Gariss prepared using additive mixture of ginger and black cumin. The fat content of Gariss prepared using an additive mixture of onion, black cumin, fenugreek and grangal (4.50± 0.34) was

significantly higher (p<0.05) compared to that prepared using additive mixtures of onion, ginger and black cumin (2.35± 0.24%), ginger and black cumin (3.25± 0.24) and onion and fenugreek (3.60± 0.15%). This might be attributed to the high oil content of these spices.

It was clear that the acidity of Gariss prepared using black cumin+ onion (2.80± 0.62%) was significantly higher (p<0.05) compared to Gariss prepared using other additives mixtures of onion+ black cumin+ fenugreek+ (0.45±0.37%),onion+ ginger+ black cumin (1.75± 0.26%); onion+ fenugreek (1.45± 0.17%) and ginger+ black cumin (1.85±0.26%) or the Gariss prepared using no additives (1.59 ±0.15%). The interviewed women herders stated that the addition of these spices improved the rate of fermentation. Furthermore, the results revealed that the types of additives had no significant effect (p<0.05) on the ash content. On the other hand, the pH value of Gariss prepared using additive mixture of onion+ ginger+ black cumin (4.55± .033%) was significantly higher (p<0.001) than that of Gariss prepared using additive mixtures of ginger+ black cumin (3.52±0.33%) and onion+ fenugreek (3.59 ±0.21%).

Table 1. Chemical composition of Gariss collected from nomadic women camel herders during dry and rainy seasons, AlGadarif State

Measurements	Dry season				Rainy season			
	Means	Min	Max	SE	Means	Min	Max	SE
Total solids (%)	11.37^{b}	13.50	10.30	0.16	13.22^{a}	12.10	14.50	0.32
Fat (%)	3.73^{a}	4.70	2.70	0.11	3.06^{b}	2.00	4.50	0.22
Protein (%)	4.88^{a}	6.38	3.70	0.14	3.97^{b}	2.30	5.10	0.27
Ash (%)	0.93^{a}	1.70	0.30	0.05	0.28^{b}	0.20	0.44	0.10
Acidity (%)	1.79^{a}	2.9	0.80	0.12	1.35^{b}	0.40	2.00	0.25
pH	3.64^{b}	5.00	2.90	0.1	4.52^{a}	3.90	5.20	0 .20

In this and the following tables:

Mean values within the same row with different superscripts letters are significantly different at p<0.05.

SE**:** Standard error

Table 2. Variation of chemical composition of Gariss prepared in different containers by nomadic camel women herders, AlGadarif State

Containers	Total solids (%) Means± SE	Fat (%) Means± SE	Acidity (%) Means± SE	Ash (%) Means± SE	pH Means± SE	Protein (%) Means± SE
Bukhsa	13.15^{a}±0.54	2.35^{c}±0.24	1.75^{a} ±0.35	0.89^{a}±0.18	4.55^{a}±0.31	4.74^{a}±0.42
Plastic	11.88^{b}± 0.27	3.65^{b}±0.12	1.47^{a} ±0.17	0.76^{a} ±0.09	3.59^{b}±0.16	4.49^{a}±0.21
Siin	11.28^{c}± 0.27	3.71^{b} ±0.12	1.867^{a} ±0.17	$0.35^{b\pm}$0.09	3.87^{ab}±0.16	4.79^{a}±0.21
Stainless steel	11.85^{bc}± 0.76	4.65^{a}±0.34	2.00^{a}±0.49	1.18^{a} ±0.25	3.95^{ab}±0.45	5.40^{a}±0.59

Table 3. Chemical composition of Gariss prepared using various additives by nomadic camel women herders, AlGadarif State

Types of containers	Total solids (%) Means± SE	Fat (%) Means± SE	Acidity (%) Means± SE	Ash (%) Means± SE	pH Means± SE	Protein (%) Means± SE
Blank	11.39^{bc} ±0.23	3.87^{ab} ±0.14	1.59^{b}±0.15	0.10^{a}±0.57	3.77^{ab} ±0.19	4.94^{a}±0.19
Black cumin+ onion	11.05^{bc} ±0.41	3.75^{abc}±0.24	2.80^{a}±0.26	0.99^{a} ±0.99	4.05^{ab} ± 0.33	4.77^{a}±0.33
Ginger+ black cumin	10.85^{c}± 0.41	3.25^{c} ± 0.24	1.85^{b}±0.26	0.60^{b}±1.40	3.52^{b}±0.33	4.47^{a} ±0.33
Onion+ fenugreek	11.93^{b}±0.26	3.60^{bc}± 0.15	1.45^{b}±0.17	0.70^{b}±0.62	3.59^{b} ±0.21	4.70^{a}±0.21
Onion+ finger+ black cumin	13.15^{a} ±0.41	2.35^{d} ±0.24	1.75^{b}±0.26	0.35^{c}±0.99	4.55^{a}±0.33	4.74^{a} ±0.33
Onion+ blak cumin+ fenugreek+ grangal	14.4^{a} ±0.58	4.5^{a}±0.34	0.45^{c}±0.37	0.20^{c}±1.40	4.05^{ab} ±0.47	2.35^{a} ±0.47

4. Discussion

Various containers are used to suit the local conditions, including seasonal movement. The seasonal systemic movement of camel herders is due to the climate conditions coupled with the lack of water (El Zubeir and Nour, 2006). The wide varieties of additives used for preparing Gariss indicating the different taste and experiences of women herders as some of the used spices were approved as having antimicrobial properties. El Zubeir *et al*. (2005) reported that black cumin, fenugreek and garlic have a significant effect on the quality of fermented milk. Moreover, the different processing methods practiced were found to influence the shelf life as presented in Figure 4. This finding supported El Zubeir and Ibrahium (2009) who concluded that pasteurization and refrigeration of camel fermented products will improve the keeping quality of the products and extending the shelf life. The variations might be due to the differences in the methods of preparation of camel's milk (Dirar, 1993; Abdelgadir *et al*., 1998; El Zubeir and Ibrahium, 2009; Ahmed *et al.*, 2010) and the storage conditions (Hassan *et al*., 2006; Hassan *et al*., 2007). Moreover, the agitation conditions under which the nomadic herders produce Gariss play a major role in the fermentation process of the product, by increasing the fermentability (Dirar, 1993; Mirghani, 1994). Finally, the temperature is found to influence the fermentative microorganisms in camel milk (Hassan *et al*., 2006).

The result for the chemical composition of Gariss agrees with that of Hassan *et al.* (2008) and Ahmed *et al.* (2010). The differences observed were attributed to the women herders' practices when separating a part of fat in order to make ghee by churning process using *Bukhsa* which reduce the fat content of the product. This was previously reported by Dirar (1993).

The Acidity of Gariss samples revealed insignificant differences among the samples collected during the two seasons, which could be due to the continuous addition of milk after the withdrawal of some Gariss, which keeps the balance of the acidity. El Zubeir and Ibrahium (2009) reported variations in developed acidity and the pH for Garris made with pasteurized and non pasteurized milk. Moreover, the variation in the chemical composition of camel milk could be due to other reasons such as the management, locations and environment (Zeleke, 2007; Bakheit *et al.*, 2008; Shuiep *et al.*, 2008; Dowelmadina *et al.*, 2014; Babiker and El Zubeir, 2014.) and processing conditions (Hassan *et al.*, 2007 and El Zubeir and Ibrahium, 2009).

The data for the pH in Gariss were similar to that of Abdelgadir *et al*. (2008); Hassan *et al*. (2008); Ahmed *et al.* (2010). This result also agrees with those reported by Dirar (1993) for Gariss samples collected from Butana area and Northern Kordofan. This might be due to the retrieving of fermented Gariss and addition of equal quantities of fresh milk, which kept the pH of the system constant (Mirghani, 1994). The data also indicated significant differences in the pH values for Gariss samples collected in different seasons, which could be explained by the storage period and quantity of previous Gariss that was used as a starter. Moreover, the acidity value also is supported by Hassan *et al*. (2008). The low pH values could be due to the type of camel milk; it might also be due to the season Shuiep *et al.*, 2008), which showed low pH during the dry season, as the camels are moved away and the frequency of adding fresh milk is prolonged. In addition, the used containers were found to affect the pH values as shown in Table 2. The low pH values of Gariss indicate major contributions of lactic acid bacteria and yeast in the fermentation. Moreover, lactic acid bacteria and yeast were reported previously as major contributing factors in the fermentation of Gariss (Dirar, 1993).

Due to the permanent movements, the nomadic camel herders need to preserve their milk for long time. Similarly Abdelgadir *et al.* (1998) reported that the pastoralists live for months depending on Gariss as their sole source of nourishment. Most of the households reported that fresh camel milk can be kept unspoiled for about 7 days. This is much longer than the shelf life of raw cows' milk; 24-48 hours; (El Zubeir, 2012). Fermented camel products generally have a longer shelf life than milk (Hassan *et al*., 2006; Hassan *et al.*, 2007; El Zubeir and Ibrahium, 2009) and are of great significance for their nutritional and social values and as a mean of generating income (Musa *et al*., 2006). Moreover, the result–showed that sometimes the camel milk may be mixed with milk from other species (mainly sheep or goat) and this is used to make porridge, and other types of cooked meal; however most of them used it alone (70%). This result supports Yagil (1982), who reported that camel milk is often mixed with fresh or churned goat milk to make milk products. A similar study in Ethiopia by Eyassu *et al*. (2007) reported that camel milk is mixed with milk of cows, goats and sheep particularly when intended to make products such as butter and cheese. El Zubeir *et al.* (2012) concluded that adding high total solids content milk of sheep to the high water content milk of camel revealed accepted yoghurt with firm texture.

5. Conclusion

The present study concluded that camel milk is consumed mainly in a fermented form (Gariss) by the nomadic camel herders. The fermentation is spontaneous using undefined bacteria at the ambient temperature. The fermentation process is uncontrollable and can result in undesirable products that shortened the product shelf life. Also the chemical constituents of Gariss were affected by the seasons (rainy and dry), types of additives and containers used in the processing. Therefore, it is recommended that training and extension should be adopted to raise awareness among producers on clean milking, handling practices for proper product quality and safety. Additional work is needed on the consistency of fermented product, effect of the additive and containers on Gariss quality.

Acknowledgements

The authors would like to thank the staff from Ministry of Animal Resources and Fisheries and the

Pastoral Union, AlGadarif State for their help during collection of the samples from the field. Special thank and appreciation for nomadic camel household keepers who provided the information and samples. The fund from Arab Science and Technology Foundation (ASTF) is appreciated with thanks.

References

Abdelgadir WS, Ahmed TK and Dirar HA. 1998. The traditional fermented milk products of the Sudan. *Inter J Food Microbiol*., **44**: 1-13.

Abdelgadir SW, Nielsen D, Hamad HS and Jakobsen M 2008. A traditional Sudanese fermented camel's milk product, Gariss, as a habitat of *Streptococcus infantarius* subsp. *infantarius*. *Inter J Food Microbiol*., **127**: 215-219.

Ahmed AI, Mohammed A A, Faye B, Blanchard B and Bakheit SA. 2010. Assessment of quality of camel milk and Gariss, North Kordofan State, Sudan. *Res J Animal and Vet Sci.,* **5**: 18-22.

AOAC. 1990a. Acidity of milk (Titrimetric method). In: Official Methods of Analysis, No. 947.05. Association of Official Analytical Chemists Inc. Virginia, USA.

AOAC. 1990b. Solids (Total) in milk. In: Official Methods of Analysis, No.925.23. Association of Official Analytical Chemists Inc. Virginia, USA.

AOAC. 1990c. Ash of milk (Gravimetric method). In: Official Methods of Analysis, No. 945. 46. Association of Official Analytical Chemists Inc. Virginia, USA.

AOAC. 1990d. Total nitrogen in milk (micro-Kjeldahl method). In: Official Methods of Analysis, No. 991.21. Association of Official Analytical Chemists Inc. Virginia, USA.

Ashmaig A, Hasan A and El Gaali E. 2009. Identification of lactic acid bacteria isolated from traditional Sudanese fermented camel's milk (Gariss). *African J Microbiol Res.,* **3(8):** 451-457.

Babiker WIA and El Zubeir IEM. 2014. Impact of husbandry, stages of lactation and parity number on milk yield and chemical composition of dromedary camel milk. *Emir. J. Food Agric*., **26 (4):** 333-341.

Bakheit SA, Majid AM and Abu-Nikhila AM. 2008. Camels (*Camelus dromedarius*) under pastoral systems in North Kordofan, Sudan: Seasonal and parity effects on milk composition. *J Camelid Sci.,* **1**: 32-36.

Bradley R L J, Arnold E J R, Barbano D M, Semerad R G, Smith D E and Viries B K. 1992. Chemical and physical methods. In: Marshall, R.T. (Ed). **Standard Methods for the Examination of Dairy Products**. American Public Health Association Washington Dc. USA.

Dirar HA. 1993. **The Indigenous Fermented Foods of the Sudan. A Study in African Food and Nutrition**. First edition, University Press, Cambridge, UNIDO C.A.B. International, Wallingford.

Dowelmadina IMM, El Zubeir IEM, Salim ADA and Arabi OHMH. 2014. Influence of some factors on composition of dromedary camel milk in Sudan. *Global Journal of Animal Scientific Research*, **2(2):** 120-129.

Elayan A A, Sulieman A E and Saleh FA. 2008. The hypo cholesterolemic effect of Gariss and Gariss containing Bifidobacteria in rats fed on a cholesterol-enriched diet. *Asian J Biochem*., **3**: 43-47.

El Zubeir IEM, Abdalla WM and El Owni OAO. 2005. Chemical composition of fermented milk (roub and mish) in Sudan. *Food Control*, **16**: 633-637.

El Zubeir IEM. 2012. Extending the shelf life of camel milk. In: E. Knoll and P. Burger (Eds.). **Camels in Asia and North Africa. Interdisciplinary Perspectives on their Past and Present Significance**. pp 219 – 224.

El Zubeir IEM, Babekir RME and Shuiep ES. 2012. Chemical properties and acceptability of yoghurt made from camel-sheep milk. The 3rd Conference of International Society of Camelids Research and Development (ISOCARD). Muscat, Sultanate of Oman, 29th January-1st February 2012.

El Zubeir IEM, Basher MAE, Alameen MH, Mohammed MAS and Shuiep ES. 2012. The processing properties, chemical characteristics and acceptability of yoghurt made from non bovine milks. *Livestock Res Rural Development* **24 (3)**.

El Zubeir IEM and Jabreel MSO. 2008. Fresh cheese from camel milk coagulated with Camifloc. *Inter J Dairy Technol*., **61**: 90-95.

El Zubeir IEM and Ibrahium MI. 2009. Effect of pasteurization of milk on the keeping quality of fermented camel milk (Gariss) in Sudan. *Livestock Res Rural Development* **21 (2).**

El Zubeir IEM and Nour EM. 2006. Studies on some camel management practices and constraints in pre-urban areas of Khartoum State, Sudan. *Inter J Dairy Sci.,* **1(2)**: 104- 112.

Eyassu S. 2007. Handling, preservation and utilization of camel milk and camel milk products in Shinile and Jijiga Zones, Eastern Ethiopia. *Livestock Res Rural Development*, **19** (6).

Hashim IB, Khalil AH and Habib H. 2008. Quality and acceptability of a set yoghurt made from camel milk. *J Dairy Sci*., **92**: 857-862.

Hassan RA, El Zubeir IEM and Babiker SA. 2006. Microbiology of camel fermented milk (Gariss) in Sudan. *Res J Microbiol*., **1(2):** 160- 165.

Hassan RA, El Zubeir IEM and Babiker SA. 2007. Effect of pasteurization of raw camel milk and storage temperature on the chemical composition of fermented camel milk. *Inter J Dairy Sci*., **2(2):** 166-171.

Hassan RA, El Zubeir IME and Babiker SA. 2008. Chemical and microbial measurements of fermented camel milk ''Garris'' from transhumance and nomadic herds in Sudan. *Australian J Basic Applied Sci*., **2**: 800- 804.

Mirghani AA. 1994. Microbiological and biochemical properties of the fermented camel milk ''Gariss''. M. Sc. Thesis, University of Khartoum, Sudan.

Musa HH, Shuiep ES and El Zubeir IEM. 2006. Camel husbandry among pastoralists in Darfur, western Sudan. *Nomadic People*, **10 (1):** 101-105.

Shori AB. 2012. Comparative study of chemical composition, isolation and identification of micro-flora in traditional fermented camel milk products: Gariss, Suusac, and Shubat. *J Saudi Soc Agricultural Sci.,* **11**: 79-88.

Shuiep ES, El Zubeir IEM, El Owni OAO and Musa HH. 2008. Influence of season and management on composition of raw camel (*Camelus dromedarius*) milk in Khartoum state, Sudan. *Tropical and Subtropical Agro-ecosystems* **8**: 101-106.

Yagil R. 1982. Camels and camel milk. Animal Production and Health Report. Rome, Italy: FAO.

Zeleke ZM. 2007. Non-genetic factors affecting milk yield and milk composition of traditionally managed camels (*Camelus dromedarius*) in Eastern Ethiopia. *Livestock Res Rural Development*, **19 (6)**.

JJBS

Jordan Journal of Biological Sciences

Volume 7, Number 2, June .2014
ISSN 1995-6673
Pages 101 - 107

Decline in Vertebrate Biodiversity in Bethlehem, Palestine

Mazin B. Qumsiyeh[1,*] , Sibylle S. Zavala[1] and Zuhair S. Amr[2]

[1] *Faculty of Science, Bethlehem University 9 Rue des Freres, Bethlehem, Palestine.*

[2] *Department of Biology, Jordan University of Science and Technology, Irbid, Jordan.*

Received: December 10, 2013 Revised: January 15, 2014 Accepted: January 20, 2014

Abstract

Our data showed that in the 1960s/1970s some 31 species of mammals and 78 species of birds were present in the area of the Bethlehem governorate, between Bethlehem and Deir Mar Saba. Comparison with observations done in 2008-2013 showed significant declines in vertebrate biodiversity in this area, which has increasingly become urbanized, with an increase in temperature and a decrease in annual rainfall over the past four decades.

Keywords: Biodiversity, Palestine, Mammals, Birds, Reptiles.

1. Introduction

Research on vertebrate biodiversity in the occupied West Bank is limited compared to that in the nearby areas of Palestine and Jordan; Palestinian research in general still lags behind (Qumsiyeh and Isaac, 2012). More work is needed to study habitat destruction including soil acidification on the fauna of the area (Graveland *et al.,* 1994; Gärdenfors *et al.,* 1995). As early as 1950, scientists warned of an environmental disaster in Palestine (Ives, 1950). The Bethlehem District covers an area of 575 km2 and includes three main towns located on the Judean hills (Bethlehem, Beit Jala, and Beit Sahour). Fertile areas lie to the west of the three towns and to the Green Line (the border between Israel and the occupied Palestinian areas). The eastern part of the area forms slow transitions from a mild Mediterranean zoogeographical region to arid habitats representing Saharo-Arabian floristic and zoo-geographical region (Zohary, 1973; Qumsiyeh, 1996). To the north is occupied Jerusalem, and to the south is the district of Hebron. In addition to the three main towns, there are 71 Palestinian smaller towns and villages, three refugee camps (since the removal of these Palestinians between 1948-1949), and 22 Israeli colonies built since the occupation of 1967 (ARIJ, 1995). The population in the district of Bethlehem tripled from roughly 95,000 in the 1960s to over 285,000 today, consisting of 205,000 Palestinians and 80,000 Jewish settlers (Palestinian Central Bureau of Statistics-PCBS, 2012). The natural increase of the native population is 3% (PCBS) and of the Israeli settler population 5% (more than twice the natural increase because more settlers were imported) (Israel Central Bureau of Statistics, 2012). Additionally, some one million tourists visit the area annually. This created a significant development in the open areas and increased the human pressure in all areas (ARIJ, 1995). However, the impact of these changes on nature was not studied.

To estimate the impact of this human development on nature is difficult. Most studies of fauna and flora of the area South of Jerusalem (Bethlehem Governorate) was done by Western visitors who came on short trips to tour the "Holy Land". One of the first native Palestinians who engaged in faunal studies was Dr. Sana Atallah who did a number of studies from 1962 until his untimely death at the age of 27 in 1970. Since 1970 until 1979, and then since 2008 until the present, the senior author has been collecting data in the area. Notes and a collection of primarily mammals and birds were accumulated in the 1960s and 1970s but only the mammal data was published (Atallah, 1977, 1978; Qumsiyeh, 1996). Because both his work in the 1960s and ours in the 1970s were focused around the Bethlehem area, they provide a baseline when compared to recent work. In sections of two books by one of the authors, the previous sparse studies were summarized, recommending that rigorous studies evaluate environmental impacts of the geopolitical changes of the past 100 years (Qumsiyeh, 1996, 2004). The example from the Hula painted frog, *Discoglossus nigriventer*, that was thought extinct after the draining of the Hula wetlands and was rediscovered recently, show the importance of these kinds of observations (Biton *et al*., 2013). In this paper, we report on vertebrate species collected or observed in this area in the 1960s and 1970s and in the past five years (2008-2013). We focus on mammals and birds but provide some data for reptiles and amphibians.

2. Material and Methods

* Corresponding author. e-mail:mazin@qumsiyeh.org.

Dr. Sana Issa Atallah was the first native Palestinian Zoologist. He was born in Beit Sahour on May, 20, 1943 and received his Master's degree from the American University of Beirut (on the rodent subfamily Microtinae) and his doctorate degree on mammals of the Eastern Mediterranean region from the University of Connecticut. He died in a car accident in Iran in January, 17, 1970. In the 1960s, he collected animals (primarily mammals but also birds, reptiles, amphibians, and even insects). Because of his frequent trips to his hometown, we had many unpublished observations on the fauna of the Bethlehem region. Only the mammal parts of these observations were included in his doctoral thesis published (with our help) posthumously (Atallah, 1977, 1978). As a teenager, the senior author accompanied him on these field trips and pursued the work after Atallah's death collecting many specimens. Thus, both Atallah and Qumsiyeh accumulated valuable specimens, mostly from Bethlehem, Beit Sahour and the valleys to the north and east. In total, specimens examined from the Bethlehem area included 62 reptiles and amphibians, 210 birds, and 150 mammals. The birds collected in the 1960s and 1970s were acquired from local children who hunted them with slingshots. The rodents were trapped and the bats were collected from caves or mist netted in wadis and agricultural areas. The mammal data was partly included in earlier works (Qumsiyeh, 1996). The data concerning birds were never published till now.

The senior author moved to the US in 1979 and returned permanently to Palestine in 2008. Between 2008-2013, we (with volunteers) studied animals in the Bethlehem area returning frequently to locations visited in the 1960s and 1970s. The present paper examines the observations and the data collected in the 1960s/1970s and comparing them to those seen more recently (2008-2013). In total, over 40 distinct collection/observation trips were made in the 1960s/1970s and 35 in the past five years covering the area. The 1960s and 1970s observations where from the eastern area of Bethlehem (roughly Mar Saba in the East and from South Jerusalem (Deir Mar Elias area) north of Bethlehem to Teqo' in the South of Bethlehem). These earlier observations did not cover the Western slopes of the Bethlehem district (a faunistically rich area). So we focused on the 2008-2013 studies on the same areas studied earlier.

Trips in the 1960s and 1970s involved collecting samples of the animals observed (including birds). We did no bird collecting during 2008-2013, but we relied on photography and observations. All vertebrate specimens collected in the two periods are housed in the Palestine Museum of Natural History (over 200 specimens are on loan to the Environmental Education Center in Beit Jala).

Field work generally followed standard procedures (RSCN, 2005). Species were identified according to Qumsiyeh (1996) for mammals, Porter *et al*. (1996) for birds, Disi *et al*. (2010) for reptiles and amphibians. In the past three years, we also added the use of an ultrasound detector to check the species of bats present in the area.

Figure 1. Area of Bethlehem covered in the present study. Note encroachment of desertification from the East and dense urbanization in the Western areas.

3. Results

Mammals

96 Species of mammals exist in Palestine (Qumsiyeh, 1996) and we recorded 31 of those in the targeted area (Table 1). But things have been changing very rapidly in this region. Thirteen of the 31 species that we noted in the 1960s and 1970s were not recorded by us in the past five years. This may even be an underestimate of the actual changes in the past century. For example, Tristram (1886) noted that *Plecotus auritus* (*Plecotus christie*) is "very common in all the hill country in Palestine especially the caves and tombs around Bethlehem and Jerusalem, and by the Sea of the Galilee" (p. 27). However, we have not noted this species even after an extensive search by using ultrasound detectors that are supposed to distinguish this species.

Out of the 31 species that were collected previously in the study area, 13 species were not recorded and four became rare during the 2008-2013 study. Only rodents including two species known as pests and associated with urbanization (*Mus musculus* and *Rattus rattus*) are still common or observed several times. In addition, the Palestinian Mole, *Spalax leucodon*, is still common. Bats were severely affected, with the absence of 4 species out of seven used to be either common or recorded several times. Similarly, species of carnivores dropped from eight to three species (Table 1). Populations of the Arabian Hare declined drastically to the level that no individuals were observed during the past five years.

Table 1. Mammal species in two different time zones.

Status: 0=Not recorded, 1=Present but rare, 2=Present (more than one observation), 3=Present and common

Family	Species	Common name	1960's-1970's	2008-2013
Erinaceidae	*Erinaceus europaeus*	European hedgehog	3	2
	Hemiechinus auritus	Long-eared hedgehog	2	0
Soricidae	*Crocidura leucodon*	Bicolored White-toothed Shrew	2	2
	Crocidura suaveolens	Lesser White-toothed Shrew	2	0
Pteropodidae	*Rousettus aegyptiacus*	Egyptian fruit bat	3	2
Rhinolophidae	*Rhinolophus blasii*	Blasius's horseshoe bat	2	0
	Rh. hipposideros	Lesser horseshoe bat	2	2
	Rh. ferrumequinum	Greater horseshoe bat	2	0
	Rh. mehelyi	Mehely's Horseshoe Bat	2	0
Vespertilionidae	*Pipistrellus kuhlii*	Kuhl's pipistrelle	3	2
	Plecotus christiei	Gray long-eared bat	1	0
Canidae	*Canis aureus*	Golden jackal	2	0
	Vulpes vulpes	Red fox	2	2
Herpestidae	*Herpestes ichneumon*	Egyptian mongoose	2	0
Hyaenidae	*Hyaena hyaena*	Striped hyena	2	1
Mustelidae	*Martes foina*	Beech marten	2	2
	Meles meles	European badger	1	0
	Mellivora capensis	Honey badger	1	0
	Vormela peregusna	Marbled polecat	1	0
Procaviidae	*Procavia capensis*	Rock hyrax	3	2
Suidae	*Sus scrofa*	Wild boar	1	2
Bovidae	*Gazella gazella*	Palestine Mountain Gazelle	3	1
Muridae	*Acomys dimidiatus*	The Eastern Spiny Mouse	3	3
	Apodemus mystacinus	Broad-toothed Field Mouse	3	2
	Dipodillus dasyurus	Wagner's Gerbil	3	1
	Meriones tristrami	Tristram's Jird	3	0
	Mus musculus	The House Mouse	3	3
	Rattus rattus	The Black Rat	2	2
Spalacidae	*Spalax ehrenbergi*	The Middle East Blind Mole Rat	3	3
Hystricidae	*Hystrix indica*	Indian Porcupine	3	1
Leporidae	*Lepus capensis*	Arabian Hare	2	0

Birds

Palestine is very rich in avifauna with over 530 species recorded. In the Bethlehem area, covered in this study, our examination of the collection maintained and notes and photographs taken by Qumsiyeh and Sana Atallah showed 78 species in the 1960s and 1970s (Table 2; Figure 2). 34 of the 78 species recorded were residents.

More recent observations over the past five years showed less than half the number of these species in the area. Twenty-nine species that were recorded earlier (1960-1970) were not observed accounting for 36% of the known species in the study area. Furthermore, invasive species such as the Hooded Crow (*Corvus corone*) appeared in the Bethlehem area.

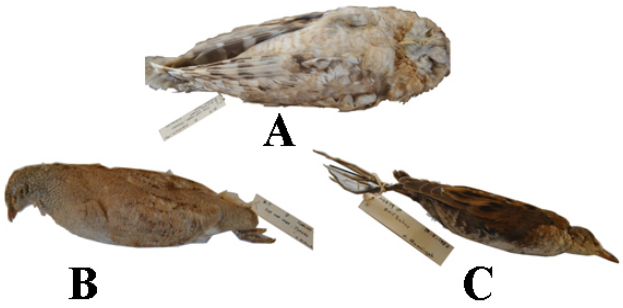

Figure 2. Three specimens of species collected in the Bethlehem area in the 1960s and 1970s. A) Female *Asio flameus*, Deir Mar Saba, 4 Feb. 1965. B) Female *Ammoperdix heyi*, Deir Mar Saba, 4 Feb. 1965; C) Male *Porzana parva*, Beit Sahour, 28 Dec. 1973.

The Yellow vented Bulbul *Pycnonotus xanthopygos* used to be, in the 19th century, more confined to the Jordan valleys or warmer wadies (see Tristram, 1884: p. 57). In the 1970s, we observed this bird, commonly in the uphill country, including the olive groves around Bethlehem in abundant numbers. Now their numbers are much less than before.

Out of the 78 species recorded from the study area, 28 species were not observed during the 2008-2013 study period, including some migrant species such as the Woodlark, Stone Curlew, Red-eyed Dove and the Yellow Hammer.

Table 2. Bird species recorded in Bethlehem district in the 1960s/70s and 2008-2013.

Status: 0=Not recorded, 1=Present, rare, 2=Present (more than one observation), 3=Present Common. M=migrant, M(WV)=migrant/winter visitor, M(PM)=migrant/passage migrant, MB=migrant and breeding, R= Resident.

Family	Species	Common name	Status	1960s-1970s	2008-2013
Accipitridae	*Aquila chrysaetos*	Golden Eagle	R	2	0
	Aquila fasciota	Bonelli´s Eagle	R	1	0
	Buteo rufinus	Long-legged Buzzard	R	2	0
Alaudidae	*Galerida cristata*	Crested lark	R	2	2
	Lullala arborea	Woodlark	M (WV)	2	0
Apodidae	*Apus apus*	Common swift	M	3	3
Burhinidae	*Burhinus oedicnemus*	Stone Curlew	M(PM)	1	0
Ciconiidae	*Ciconia ciconia*	White stork	M(PM)	2	2
Columbidae	*Columba livia*	Rock dove	R	3	3
	Streptopelia decaocto	Collared dove	R	2	2
	Streptopelia roseogrisea	African collared dove	R	2	1
	Streptopelia senegalensis	Palm dove	R	1	0
	Streptopelia semitorquata	Red eyed dove	M	2	0
	Streptopelia turtur	Turtle dove	M(PM)	3	2
Corvidae	*Corvus corax*	Raven	R	1	1
	Corvus corone	Hooded Crow	R	0	3
	Garrulus galandarius	Eurasian Jay	R	3	3
Emberizidae	*Emberiza calandra*	Corn Bunting	M(WV)	2	1
	Emberiza citrinella	Yellow Hammer	M(WV)	2	0
	Emberiza hortulana	Ortolan Bunting	M(PM)	2	1
Fringillidae	*Carduelis cannabina*	Linnet	R	3	1
	Carduelis carduelis	Goldfinch	R	3	0
	Carduelis chloris	Greenfinch	R	3	2
	Fringilla coelebs	Chaffinch	M(WV)	2	1
	Fringilla montifringilla	Brambling	M(WV)	1	1
	Carduelis spinus	Siskin	M(WV)	2	2
	Serinus serinus	Serin	M(WV)	2	0
Falconidae	*Falco tinunculus*	Kestrel	R	3	2
Hirundinidae	*Ptyonoprogne fuligula*	Rock Martin	R	2	1
	Ptyonoprogne rupestris	Pale Crag Martin	M(WV)	2	0
Laniidae	*Lanius excubitor*	Great Grey Shrike	R	2	1
	Lanius minor	Lesser Grey Shrike	M(PM)	2	1
	Lanius nubicus	Masked Shrike	MB	3	2
	Lanius senator	Woodchat shrike	MB	2	1
Motacillidae	*Motacilla alba*	White wagtail	M(WV)	2	2
	Motacilla flava	Yellow Wagtail	M(PM)	2	2

Muscicapidae	*Muscicapa striata*	Spotted Flycatcher	MB	2	1
Nectariniidae	*Nectarinia osea*	Orange-tufted Sunbird	R	3	1
Paridae	*Parus major*	Great Tit	R	3	2
Phasianidae	*Alectoris chukar*	Chukar Partridge	R	3	2
	Ammoperdix heyi	Sand Partridge	R	2	0
	Coturnix coturnix	Common Quail	M(PM)	2	0
Picidae	*Dendrocopos syriacus*	Syrian woodpecker	R	2	0
	Jynx torquilla	Wryneck	M(PM)	2	0
Ploceidae	*Passer domesticus*	House sparrow	R	3	3
Ploceidae	*Petronia petronia*	Rock Sparrow	R	2	0
Prunellidae	*Prunella modularis*	Dunnock	M(WV)	2	0
Pycnonotidae	*Pycnonotus xanthopygos*	Yellow-vented Bulbul	R	3	2
Rallidae	*Porzana parva*	Little crake	M(PM)	1	0
Strigidae	*Asio flammeus*	Short-eared owl	M(PM)	1	0
	Athene noctua	Little owl	R	3	2
	Bubo bubo	Eagle owl	R	1	1
	Otus scops	Scops owl	M(PM)	3	0
Sturnidae	*Sturnus vulgaris*	Common Starling	M(WV)	2	1
Sylviidae	*Cettia cetti*	Cetti´s Warbler	R	2	1
	Hippolais pallida	Olivaceous Warbler	MB	1	0
	Hippolais olivetorum	Olive-tree Warbler	M(PM)	1	0
	Locustella luscinoides	Savi´s Warbler	M(PM)	1	1
	Phylloscopus sibilatrix	Wood Warbler	M(PM)	2	1
	Sylvia atricapilla	Blackcap	M	3	3
	Sylvia curruca	Lesser Whitethroat	M(PM)	3	2
	Sylvia communis	Common Whitethroat	M(PM)	2	2
	Sylvia melanocephala	Sardinian Warbler	R	2	1
Turdidae	*Cercomela melanura*	Blackstart	R	2	1
	Cercotrichus galactotes	Rufous Bushchat	M	2	0
	Erithacus rubecula	Robin	M(PM)	1	0
	Luscinia megarhynchos	Nightingale	M(PM)	2	0
	Oenanthe hispanica	Black-eared Wheatear	M(PM)	3	2
	Oenanthe isabellina	Isabelline Wheatear	MB	2	1
	Oenanthe lugens	Mourning Wheatear	R	2	1
	Oenanthe oenanthe	Eurasian Wheatear	M(PM)	2	2
	Phoenicurus phoenicurus	Common Redstart	M(PM)	5	0
	Phoenicurus ochruros	Black Redstart	M(WV)	2	1
	Saxicola torquata	Common stonechat	M(WV)	2	1
	Turdus philomelas	Song thrush	M(WV	3	0
	Turdus merula	Blackbird	R	3	0
Tytonidae	*Tyto alba*	Barn owl	R	3	1
Upupidae	*Upupa epops*	Hoopoe	MB	3	2

Reptiles and Amphibians

Unlike birds and mammals, we did not perform a complete systematic survey of reptiles and amphibians in the Bethlehem area. We had few observations which are worth mentioning since no previous studies were reported on the herpetofauna of this area. Reptiles we observed and/or collected in the 1960s and 1970s, including *Hemorrhois nummifer* (3 specimens collected on 2.5.1964, and 10.6.1966), *Dolichophis jugularis* (one specimen collected in May 1974), *Stellagama stellio* (dozens of observations and several specimens throughout the year except winter), *Chameleo chameleon*, *Ptyodactylus guttatus* (5 specimens, PMNH R8, on 7.9.1964), *Hemidactylus* sp. (PMNH-R44, on 11.7.1966; PMNH-R57, on 8.10.1976), *Testudo graeca* (common),

Rhynchocalamus melanocephalus (2 specimens, Beit Sahour, in April 1974), *Trachylepis vittata* (4 km NW Beit fajjar 2 specimens PMNH-R34, on 29.3.1966), *Phoenicolacerta laevis* (several observations in the 1970s, one specimen PMNH-R77, on 17.6.1966), *Ophisops elegans, Eumeces schneideri* (Beit Sahour, on 6.5.1973), and *Acanthodactylus* sp. (Beit Sahour and Deir Ibn Ubeid, several specimens). We observed all these species in the period 2008-2013. Although these are rather casual observations, they could suggest that reptiles were less vulnerable to the environmental/habitat changes seen here than mammals and birds (see discussion).

However, with amphibians, another story is noted. The Tree Frog, *Hyla savignyi*, was rather common in the areas of Solomons' pools and Artas and declined rapidly over the past few decades. It still occurs in Husan and Battir areas though in small numbers (Salman and Qumsiyeh, In press). In late 2000s, the Islamic Waqf leased the Solomon's pools to developers who established the Bethlehem Convention Center with its various amenities there (opened in 2009). In 2010, the business operators drained and "cleaned" the pools. We have recently reintroduced some tree frogs there (obtained from the nearby area of Wadi Fukeen). The toad *Pseudepidalea viridis* was extremely common in the district in the 1970s. One could observe dozens of these toads everywhere in the fields and even the streets of towns like Beit Sahour and Ubaidya after a good rain. The small winter ponds in the valleys surrounding Jebel Abu Ghnaim teamed with these toads in winter time. That area has ever since been transformed to a large Jewish settlement called Har Homa. Many of the bird species we observed in the 1970s were also observed around Jabal Abu Ghneim.

4. Discussion

Our study is certainly not a comprehensive sampling of the local fauna; but our methods in the 1960s and 1970s are similar to our methods in the many trips during the past 5 years. Thus while perhaps several species could have been missed, the overall picture of the decline we documented is significant especially with regards to mammals, birds and amphibians. Further studies on reptiles are needed. If we treat the data as statistically recorded/not recorded, we find that there was a drop from 31 species to 18 species of mammals, and a drop from 78 species to 50 species of birds (Tables 1 and 2). Amphibians have also declined markedly in the targeted area. The significant decline in vertebrate biodiversity in the Eastern slopes of the Bethlehem district over the past few decades is alarming. Local people lived in harmony with nature for millennia except for a few documented cases of the overuse of the environment; for example in Ain Ghazal in Jordan (Kahler-Rollefson and Rollefson, 1990). The more dramatic changes witnessed in the past 100-150 years are exceptional. The most notable, via looking at statistics and satellite image, is the rampant human population growth and urbanization of the Bethlehem area. As noted, the human population more than tripled between the 1960s and 2008-2013. Land cover changes has also been spectacular. The forested hill of Jabal Abu Ghneim, for example, was transformed to an urban Israeli colony called Har Homa. But other changes are also significant including pronounced impact of global warming (Evans, 2009). The World Bank report in November 2012 on the impact of human induced climate change on the Arab world revealed unsustainable trends. Over the past 20 years, climate monitoring stations across the Arab world have already shown an increase in average annual temperature. Computer models predict that in the next two to three decades annual rainfall will decrease in our area by nearly 25% and average annual temperatures will climb by 4-5 degrees. Climate change makes things far worse because of changes that will impact habitats due to unfamiliar rain patterns (Alpert *et al*., 2002) and the way it will interacts with other issues like urbanization and population shifts (IPCC, 2007; Qumsiyeh, 2013).

Disentangling the causes of declines can be straight forward in some cases and more complicated in others. For example, it is obvious that the Israeli systematic fumigation of caves caused a decline in local bat biodiversity (Makin and Mendelsohn, 1987; Qumsiyeh, 1996; Korine *et al*., 1999). In other cases, it may not be obvious. Yom-Tov (2001) suggested that the decline in the body mass of four species of birds between the 1950s and 1999 is due to global climatic change. Per Bergman's rule, higher temperature can lead to micro-evolutionary changes producing smaller size. But phenotypic plasticity may also play a role in this case (Teplitsky *et al*., 2008) as are other changes in the environment/resource availability. Bilgin *et al*. (2012) used two models and concluded that bat species will be most significantly affected in our area due to climate change. However, caution must be taken in putting out predictive models about the effect of climate change on biodiversity because models cannot take into consideration issues like topography, microclimates, and individual species adaptability (Willis and Bhagwat, 2009). We thus feel that direct studies, like the preliminary study we presented above, are important for monitoring changes in biodiversity.

In the case of the Bethlehem district, we have to consider the geopolitical and population changes. Between 1949-1950, Bethlehem received an influx of refugees removed from their lands to create the Jewish state of Israel. Initially over 50,000 refugees settled in the Bethlehem district but then many moved out. Today, there are over 55,000 of those refugees and their descendants living in the district for a total Palestinian population of the district of 205,000. Since 1967 Israel has also built over 20 settlements in Bethlehem area that now house over 80,000 settlers (ARIJ, 2007).The settlement of Har Homa was built on the previously forested area of Jebel Abu Ghneim between Beit Sahour and Jerusalem which necessitated the destruction of 60,000 trees (ARIJ 2007; see also Qumsiyeh, 2001). The destruction of an environmentally sustainable way of life of the native people forced them to live as semi-urban dwellers in refugee camps or in sprawling suburbs at the edges of large cities (Qumsiyeh, 2004; Qumsiyeh and Issac, 2012). It is these areas that were especially affected by environmental damages and that now include a wall that clearly impacts the environment (Reese, 2003). By

fragmenting habitats, the wall reduces population size and prevents normal movement of wildlife. The wall disrupts the contiguity of natural water flows of streams and springs and this affects vegetation in the area thus effecting fauna.

Similar problems are likely in other areas of historic Palestine and in the nearby areas, like Jordan, where demographic changes have been spectacular in the last few decades. In some other areas, peculiar problems are noted. For example, the draining of the Hula wetlands in the 1950s by the then nascent state of Israel and the diversion of the water from the Jordan River basin created huge environmental problems. 97% of the wetlands in Palestine were drained by Israeli authorities. The idea of large scale manipulation of the Palestinian environment started under the British mandate to fulfill the Zionist ideological desires of transforming the land and the people (Sufian, 2007) but is now compounded by population growth, limited resources and global warming (Qumsiyeh, 2013).

Acknowledgement

This work is dedicated to the late Zoologist Dr. Sana Atallah (1943-1970) whose specimens and field notes from the 1960s formed a significant part of this work. For field work in the 1970s, we are grateful to Walid Qumsiyeh and Jad Isaaq, and for that in the past five years to Ibrahim Salman, Michel Salsaa and Jessie Chang.

References

Alpert P, Ben-Gai T, Bahrahad A, Benjamini Y, Yekutieli D, Colacino M, Diodato L, Ramis C, Homar V, Romero R, Michaelides S and Manes A. 2002. The paradoxical increase of Mediterranean extreme daily rainfall in spite of decrease in total values. *Geophysical Research Letters* **29**(11): doi:10.1029/2001GL013554.

ARIJ (Applied Research Institute of Jerusalem). 1995. Environmental of the West Bank. I. Bethlehem District. Published by ARIJ, Bethlehem.

ARIJ (Applied Research Institute of Jerusalem). 2007. Geopolitical Status of Bethlehem Governorate, 2007. Published by ARIJ, Bethlehem.

Atallah Sl. 1977. Mammals of the Eastern Mediterranean Region; their ecology, systematics and zoogeographical relationships. S*äugetierkundliche Mitteilungen*, **25**:241-319.

Atallah SI. 1978. Mammals of the Eastern Mediterranean Region; their ecology, systematics and zoogeographical relationships. *Säugetierkundliche Mitteilungen*, **26**:1-50.

Bilgin R, Keşişoğlu A and Rebelo H. 2012. Distribution patterns of bats in the eastern Mediterranean region through a climate change perspective. *Acta Chiropterologica*, **14**:425-437.

Biton R, Geffen E, Vences M, Cohen O, Bailon S, Rabinovich R, Malka Y, Oron T, Boistel R, Brumfeld V and Gafny S. 2013. The rediscovered Hula painted frog is a living fossil. *Nature Communications*, **4**(1959):1-6.

Evans JP. 2009. 21st century climate change in the Middle East. *Climate Change*, **92**:417-432.

Gärdenfors U, Waldén HW and Wäreborn I. 1995. Effects of soil acidification on forest land snails. *Ecol Bulletins*, **44**: 259-270.

Graveland J, van der Wal R, van Balen JH and van Noordwijk AJ. 1994. Poor reproduction in forest passerines from decline of snail abundance on acidified soils. *Nature*, **368**: 446–448.

IPCC (Intergovernmental Panel on Climate Change) 2007. Working Group I Report - The Physical Science Basis. Available at: http://www.ipcc.ch/ipccreports/ar4-wg1.htm.

Ives R. 1950. The Palestinian environment. *Am Scientist*, **38**:85-104.

Kahler-Rollefson I and Rollefson G. 1990. The impact of Neolithic subsistence strategies on the environment: the case of 'Ain Ghazal, Jordan. In: Bottema S, Entjes-Nieborg G and Van Zeist W. (Eds.); **Man's Role in the Shaping of the Eastern Mediterranean Landscape**. Balkema Press, Rotterdam, 3-14.

Korine C, Izhaki I and Arad Z. 1999. Is the Egyptian fruit-bat *Rousettus aegyptiacus* a pest in Israel? An analysis of the bat's diet and implications for its conservation. *Biol Conservation*, **88**:301-306.

Makin D and Mendelssohn H. 1987. Israel wipes out its bats. *Ecologist*, **16**:1-2.

Qumsiyeh MB. 1996. **Mammals of the Holy Land**. Texas Tech Press, Lubbock.

Qumsiyeh MB. 2004. **Sharing the Land of Canaan: Human Rights and the Israeli-Palestinian Struggle**. Pluto Press.

Qumsiyeh MB and Isaac J. 2012. Research and development in the Occupied Palestinian Territories: challenges and opportunities. *Arab Studies Quarterly*, **34**:158-172.

Qumsiyeh MB. 2013. The Coming Environmental Nakba. The Third Palestinian Environmental Awareness and Education, Conference. EEC, Bethlehem. Pp 57-59.

Reese J. 2003. Israel's Apartheid Wall: Environmental Disaster in Palestine. Earth First Journal. http://www.earthfirstjournal.org/article.php?id=161.

Salman I and Qumsiyeh MB. Distribution and cytogenetics of amphibians from the occupied Palestinian territories (West Bank of Jordan). *Jordan J Natural History (In press)*.

Sufian SM. 2007. **Healing the Land and the Nation: Malaria and the Zionist Project in Palestine**, 1920-1947. University of Chicago Press.

Teplitsky C, Mills J.A, Alho JS, Yarrall JW and Merilä J. 2008. Bergmann's rule and climate change revisited: Disentangling environmental and genetic responses in a wild bird population. *Proceedings of the National Acad Sci., (USA)*, **105**(36): 13492-13496**.**

Tristram HB. 1866. Report on the mammals of Palestine. *Proceedings of the Zool Soc London*, **1866**:84-93.

Tristram HB. 1884: **The Survey of Western Palestine. The Fauna and Flora of Palestine.** London: The Committee of the Palestine Exploration Fund, xxii+455 pp.

Willis KJ and Bhagwat SA. 2009. Biodiversity and climate change. *Science*, **326**:806–807.

Yom-Tov Y. 2001. Global warming and body mass decline in Israeli passerine birds. *Proceedings of the Zool Soc London*, **268**:947-952.

Zohary M. 1973. **Geobotanical Foundations of the Middle East.** B. Fischer Verlag, Stuttgart, 739 pp.

JJBS

Jordan Journal of Biological Sciences

Volume 7, Number 2, June .2014
ISSN 1995-6673
Pages 109 - 112

Diet of the Barn Owl (*Tyto alba*) from Chaddra-Akkar, Northern Lebanon

Mounir R.Abi-Said[1,*], Adwan H.Shehab[2] and Zuhair S.Amr[3]

[1] *Faculty of Sciences II, Lebanese University, Al-Fanar, Lebanon and Animal Encounter, Ras Al-Jabal, Aley,Lebanon;*

[2] *General Commission for Scientific Agricultural Research, Douma, P.O. Box 113, Damascus, Syria;*

[3] *Department of Biology, Jordan University of Science and Technology, Irbid, Jordan.*

Received: December 10, 2013 *Revised: January 16, 2014* *Accepted: January 25, 2014*

Abstract

Pellets, regurgitated by the Barn Owl, *Tyto alba*, were collected from Chadra-Akkar region in northern Lebanon. Pellets analyses yielded remains of 249 individuals, representing nine mammalian species and one species of a passerine bird. Small mammals constituted 96.4% of its diet (90.4% rodents and 6.0% insectivores), while birds constituted 3.6%. Günther's Vole, *Microtus guentheri*, and the House Mouse, *Mus domesticus/macedonicus*, were the most abundant prey items, representing 34.4% and 32.1% of the total number of recovered prey items respectively. The number of prey items per one pellet ranged from 1-6 individuals (Average 2.15 ±1.23 skull/pellet, N= 55).

Keywords: Barn Owl, *Tyto alba*, Owl pellets, Small mammals, Rodentia, Lebanon.

1. Introduction

Owl pellets have been studied on a few occasions in Lebanon. Bate (1945) recoded eight species of mammals recovered from the Long-eared Owl, *Asio otus*, pellets collected form Cedars of Bsherreh. Bayle and Prior (2006) examined pellets of the Euroasian Eagle Owl, *Bubo bubo*, that included cranial remains for mammals, reptiles and birds in addition to insects from Qab Elias. Obuch and Benda (2009) reported on remains recovered from the Barn Owl, *Tyto alba*, from Sour and Saida, southern Lebanon.

Pellet analyses of Barn Owl, *T. alba*, in the Middle East was investigated by several authors (Dor, 1947; Hoppe, 1986; Kasparek, 1988; Brinkmann *et al.*, 1990; Rekasi and Hovel, 1997; Rifai *et al*., 1998; Pokines and Peterhans, 1998; Abu Baker *et al*., 2005; Pokines *et al*., 2011).

Owl pellets investigation is a quick method to report on the diversity of small mammals. Many recent studies have been published in the neighboring countries to report on the diversity of small mammals from owl pellets. Reports on the diversity of small mammals in Lebanon has been scarce during the last few decades, with few that have been published recently (Abi-Said, 2004 and 2009; Abi-Said and Kryštufek, 2012).

In this account, we report on the diet composition of the Barn Owl in the Chaddra-Akkar region, northern Lebanon.

2. Material and Methods

The material of this study consists of intact pellets and pellets' fragments regurgitated by the Barn Owl, *Tyto alba*, collected in December 2012 from the ground under a rock shelter from Chaddra-Akkar region (34^{o} 37'.327" N, 36^{o} 19'.231" E). The pellets were found in a typical Mediterranean forest dominated by oak trees (*Quercus* sp.). Most of the pellets had accumulated a long time ago and this was evident from the presence of moth moulting cocoons on the pellets while few were fresh.

A total of 55 intact pellets, besides many incomplete pellets, were collected. Each pellet was soaked in water for few seconds. Cranial remains (skulls and mandibles) were removed and kept separately. The identification was based on cranial elements, and the number of individuals was determined based on the highest number of cranial elements using reference specimens deposited at the Natural History Museum at the American University of Beirut.

3. Results and Discussion

The examined material yielded a total of 249 individual prey items (Table 1). By species, seven, two and one species of rodents, insectivores and passerine bird, were recovered, respectively. Small mammals were the main prey items accounting for 96.4% of the diet (rodents 90.4% and shrews 6.0%), while birds represented 3.6% of the diet. The number of prey items

* Corresponding author. e-mail: mabisaid9@gmail.com.

per pellet ranged from 1-6 individuals (average 2.15 ±1.23 skull/pellet, N= 55).

Table 1. Summary for prey items consumed by the Barn Owl in Chaddra-Akkar, Lebanon.

Species	No. of individuals	%
Order Rodentia		
Apodemus mystacinus	14	5.6
Mus domesticus/macedonicus	80	32.1
Cricetulus migratorius	6	2.4
Microtus guentheri	108	43.4
Meriones tristrami	9	3.6
Dipodillus dasyurus	7	2.9
Spalax ehrenbergi	1	0.4
Order Soricomorpha		
Crocidura suaveolens	13	5.2
Suncus etruscus	2	0.8
Aves		
Passeriformes sp.	9	3.6
Total	249	100

Order Rodentia

Seven rodent species belonging to three families were identified (Muridae: *Apodemus mystacinus*, *Dipodillus dasyurus, Meriones tristrami, Mus domesticus/macedonicus;* Cricetidae: *Cricetulus migratorius, Microtus guentheri*; Spalacidae: *Spalax ehrenbergi*).

Family Muridae

Apodemus mystacinus (Danford & Alston 1877)

Remains of 14 skulls of the Eastern Broad-toothed Field Mouse were recovered. Shehab (2005) recovered this species from *T. alba* pellets from, Kharabow, southern Syria, and from Qala'at al Hosn, Qala'at Salah ad Din and Qala'at Sheisar in Syria (Obuch and Benda, 2009).Cranial measurements and teeth structure are in agreement with Harrison and Bates (1991) and Kryštufek and Vohralík (2009). Bayle and Prior (2006) recovered *A. mystacinus* from *B. bubo* pellets near Qab Elias, Lebanon.

Dipodillus dasyurus (Wagner, 1842)

Seven skulls of Wagner's Dipodil were recovered from the collected pellets accounting for 2.9%. This species is not very common in Lebanon; it was recorded by Abi-Said (2009) from Jabal Moussa- Biosphere Reserve. Pellets of *T. alba* contained remains of this species in Jordan and the Negve desert (Rekasi and Hovel, 1997; Abu Baker *et al*., 2005). Collected from pellets of Desert Eagle Owl, *Bubo bubo ascalaphus* (Rifai *et al*., 2000), and the Little Owl, *Athene noctua*, from the eastern deserts of Jordan (Al-Melhim *et al*., 1997).

Meriones tristrami Thomas, 1892

The nine skulls of Tristram's Jird were recovered representing 3.6%. It is found to be common in *T. alba* pellets collected from Sour, southern Lebanon and localities in western Syria (Obuch and Benda, 2009). Bate (1945) recovered *M. tristrami* remains from *A. otus* pellets collected from Cedars of Bsherreh. Bayle and Prior (2006) recovered this species from *B. bubo* pellets near Qab Elias, Lebanon. Cranial measurements and skull morphology agreed with those given by many authors (Lewis *et al*., 1967; Atallah, 1978; Tohmé and Tohmé, 1985, Harrison and Bates, 1991; Kryštufek and Vohralík, 2009).

Mus domesticus/macedonicus

The Western House Mouse/Macedonian House Mouse ranked second in the diet of the owl, where 80 skulls of this species were recovered representing 32.1% of the total number of prey items. The largest number of skulls per pellet (6 skulls) was found for this species. Similar maximum number of *Mus* sp. in diet of Barn Owl was recorded in Syria (Shehab, 2005) and from Shaumari Wildlife Reserve, Eastern Jordan (Abu Baker *et al*., 2005). Other authors indicated that this species is the most frequent food item of the Barn Owl in the Middle East (Nader, 1968; Hoppe, 1986; Kasparek, 1988; Brinkmann *et al*., 1990). The *Mus* sp. is a commensal and invasive species that can establish colonies around human settlements.

Family Cricetidae

Cricetulus migratorius (Pallas, 1773)

Six skulls of the Grey Dwarf Hamster were recovered, constituting 2.4%. Seeds were observed on the surface of those pellets containing remains of the Grey Dwarf Hamster. Such a finding was indicated from owl pellets containing *C. migratorius* remains from Syria (Shehab *et al.*, 1999). Cheek pouches of the Grey Dwarf Hamster are usually filled with seeds while foraging, explaining their occurrence in pellets (Shehab, 2005). This species was recovered from *T. alba* pellets collected from ar'Rasafeh, Syria (Shehab *et al*., 2004) and other localities in western Syria (Obuch and Benda, 2009).

Microtus guentheri (Danford & Alston, 1880)

Günther's Vole was the most common prey item found, with a total of 108 skulls recovered constituting 43.4%. This perhaps suggests that voles are very abundant in the study area, and indicates that owls play a vital role in regulating voles' populations in this area. It was recovered from *T. alba* pellets from several locations in Syria (Shehab *et al*., 2004; Obuch and Benda, 2009) and Jordan (Rifai *et al*., 1998). Pellets recovered from *T. alba* from Saida, southern Lebanon, are characterized by the complete absence of Günther's Vole remains (Obuch and Benda, 2009). Bate (1945) recovered Günther's Vole remains from *A. otus* pellets collected from Cedars of Bsherreh. Bayle and Prior (2006) recovered *Microtus socialis* (=*Microtus*

guentheri) from *B. bubo* pellets near Qab Elias, Lebanon.

Family Spalacidae

Spalax ehrenbergi (Nehring, 1898)

Only one skull of the Palestinian Mole Rat was found (0.4% of the diet). Similarly, Obuch and Benda (2009) found one skull in southern Lebanon in *T. alba* pellets, and Bayle and Prior (2006) recovered one single specimen of this mole from *B. bubo* pellets near Qab Elias, Lebanon. The presence of this species in very low numbers in the owl's diet does not mean that this species is rare, but is due to the fossorial lifestyle, thus avoiding predation.

Order Soricomorpha

Family Soricidae

Crocidura suaveolens (Pallas, 1811)

Thirteen skulls of Lesser White-toothed Shrew were found, representing 5.2%. Previously recorded from *T. alba* pellets were collected from Sida and Sour (Obuch and Benda, 2009). Remains of the Lesser White-toothed Shrew were recovered from *T. alba* pellets collected in Jordan and Syria (Rifai *et al*., 1998; Abu Baker *et al*., 2005; Shehab, 2005; Shehab and Al Charabi, 2006). Bate (1945) found remains of *Crocidura russula* in *A. otus* remains collected from Cedars of Bsherreh.

The cranial measurements, skull and teeth structure are in agreement with the specimens of *C. suaveolens* collected by Sana Atallah, kept at the mammalian collection at the Natural History Museum of the American University of Beirut.

Suncus etruscus (Savi, 1822)

Only two skulls of the Pygmy White-toothed Shrew were recovered. Similar results were obtained from pellets of *T. alba*, collected from southern Lebanon, Jordan and Syria (Abu Baker *et al*., 2005; Shehab, 2005; Shehab and Al Charabi, 2006; Obuch and Benda, 2009).

Birds

Nine skulls of one species of a passerine bird were found, representing 3.6% of the total number of the prey items.

4. Conclusion

Owls are generally opportunistic feeders and rely on available food resources within their home range. Variable food items are consumed depending on the synchrony of owl's activity and the prey. *Tyto alba* is a nocturnal species that feeds on nocturnal small-sized animals including mammals, birds as well as some insects. In this study, most small mammals that were consumed by the Barn Owl, expect *Spalax ehrenbergi*, are nocturnal. This explains the low number of consumed rat moles. Amount of small nocturnal mammals in pellets may very well provide an estimate to their spatial abundance in a particular habitat inhabited by the Barn Owl.

References

Abi-Said MR. 2004. First record of the Five-toed Jerboa, *Allactaga euphratica*, Thomas, 1881 in Lebanon. *Zool Middle East*, **33**:149-152.

Abi-Said MR. 2009. First record of Wagner's Gerbil, *Gerbillus dasyurus* (Wagner, 1842) (Mammalia: Rodentia), in Lebanon. *Zool Middle East*, **48**:101-102.

Abi-Said MR and Kryštufek B. 2012. First record of the Forest Dormouse, *Dryomys nitedula* (Pallas, 1799), in Lebanon (Mammalia: Rodentia). *Zool Middle East*, **56**:137-139.

Abu Baker M, Qarqaz M and Amr Z. 2005. Small mammal remains recovered from pellets of the Barn Owl *(Tyto alba*) at Shaumari Wildlife Reserve, eastern Jordan. *Časopis Národního Muzea, Rada prirodovedná*, **174**:127-129.

Al-Melhim WN, Amr ZS, Disi AM and Katbeh-Bader A. 1997. On the diet of the Little Owl, *Athene noctua*, from Safawi area, eastern Jordan. *Zool Middle East*, **15**:19-28.

Atallah SI. 1978. Mammals of Eastern Mediterranean region; their ecology, systematic and zoogeographical relationships. Part 2. *Säugetierkundliche Mitteilungen*, **26**:1-50.

Bate DMA. 1945. Notes on small mammals from the Lebanon Mountains, Syria. *J Natural History, Series 11*, **12**:141–158.

Bayle P and Prior R. 2006. Prey species of Eagle Owl *Bubo bubo* in Lebanon. *Sandgrouse*, **28**:167-168.

Brinkmann R, Heins JU, Köhler B and Rösler S. 1990. **Menderes-Delta. Zustand und Gefährdung eines ostmediterranen Flußdeltas**. Kassel: Gesamthoschule Kassel, xiv + 241 pp.

Dor M. 1947. Observations sur les micromammifères trouvés dans les pelotes de la chouette effraye (*Tyto alba*), en Palestine. Mammalia, **11**:49-54.

Harrison DL and Bates PJJ. 1991. **The Mammals of Arabia**. 2nd ed. Harrison Zoological Museum Kent, England, xv + 354 pp.

Hoppe N. 1986: Pellets contents of the Barn owl, *Tyto alba*, near Samandag, Turkey. *Zool Middle East*, **1**: 29-32.

Kasparek M. 1988: **Der Bafasee. Natur und Geschichte in der türkischen Ägäis**. Heidelberg: Max Kasparek, 174 pp.

Kryštufek B and Voharlík V. 2009. **Mammals of Turkey and Cyprus. Rodentia II: Cricetinae, Muridae, Spalacidae, Calomyscidae, Capromyidae, Hystricidae, Castoridae.** University of Primorska, Science and Research Centre Koper. 371 pp.

Lewis RE, Lewis JH and Atallah SI. 1967. A review of Lebanese mammals. Lagomorpha and Rodentia. *J Zool*., **153**:45–70.

Nader I. 1968. Animal remains in pellets of the Barn Owl, *Tyto alba*, from the vicinity of An-Najaf, Iraq. *Bulletin of the Iraq Natural History Museum*, **4**:1-7.

Obuch J and Benda P. 2009. Food of the Barn Owl (*Tyto alba*) in the Eastern Mediterranean. *Slovak Raptor J*., **3**:41-50.

Pokines JT and Peterhans JCK. 1998. Barn Owl *(Tyto alba)* taphonomy in the Negev Desert, Israel. *Israel J Zool*, **44**: 19-27.

Pokines JT, Nowell A, Bisson MS, Cordova CE and Ames CJH. 2011. The functioning of a natural faunal trap in a semi-arid environment: preliminary investigations of WZM-1, a

limestone sinkhole site near Wadi Zarqa Ma'in, Hashemite Kingdom of Jordan. *J Taphonomy*, **9**:89-115.

Rekasi J and Hovel H. 1997. Pellet contents of barn owls *Tyta alba erlanger;* from Be'erotayim, Israel. *Israel J Zool.*, **43**:305-306.

Rifai L, Al-Melhim W and Amr Z. 1998. On the diet of the Barn Owl, *Tyto alba*, in Northern Jordan. *Zool Middle East*, **16**:31-34.

Rifai LB, Al-Melhim WN, Gharaibeh BM and Amr Z. 2000. The diet of the Desert Eagle Owl, *Bubo bubo ascalaphus*, in the Eastern Desert of Jordan. *J Arid Environ.*, **44**:369-372.

Shehab AH and Al Charabi SM. 2006. Food of the Barn Owl *Tyto alba* in the Yahmool Area, Northern Syria. *Turkish J Zool.*, **30**:175-179.

Shehab AH, Kowalski K and Daoud A. 1999. Biometrical remarks on the Golden Hamster *Mesocricetus auratus* (Waterhouse, 1839) (Cricetidae, Rodentia) from Ebla (Northern Syria). *Acta Zool Cracoviensia*, **42**:403-406.

Shehab AH, Daoud A, Kock D and Amr Z. 2004. Small mammals recovered from owl pellets from Syria (Mammalia: Soricidae, Chiroptera, Rodentia). *Zool Middle East*, **33**:27-42.

Shehab AH. 2005. Food of the Barn owl *Tyto alba* in Southern Syria. *Acta Zool Cracoviensia*, **48A**:35-42.

Tohmé G and Tohmé H. 1985. **Les Mammiferes Sauvages Du Liban**. Publications de l'Universite Libanaise, Beirut, Lebanon.

JJBS

Volume 7, Number 2, June .2014
ISSN 1995-6673
Pages 113 - 118

Jordan Journal of Biological Sciences

Effect of *Hiptage madablota* Gaertn. on High Fat Diet – Induced Obese Rats

Gandhimathi Retnasamy* and Sreedevi Adikay

Institute of Pharmaceutical Technology, Sri Padmavati Mahila Visvavidyalayam, Women's University, Tirupati, Andhra Pradesh – 517502, India.

Received: December 7, 2013 Revised: February 7, 2014 Accepted: February 13, 2014

Abstract

The present study is designed to evaluate theanti-obesity activity of the roots of *Hiptage madablota* Gaertn against high fat diet-induced obese rats. In the ethanolic extract of *Hiptage madablota* (EEHM) total saponin content is also estimated by gravimetric method. Obesity is induced in albino rats by the administration of high fat diet for 40 days. Therefore, this study is accentuated to explore the efficacy of the ethanol extract of *Hiptage madablota* root at the dose of 100, 200 and 400mg/kg by oral. The anti-obesity activity is estimated in terms of food intake, body weight, lee index, serum lipids, atherogenic index, coronary risk index and brain serotonin level in rats. Preliminary phytoconstituents analysis revealed the presence of flavonoids, terpenoids, saponins, phenolic compounds and tannins. Animal received oral EEHM (100, 200 &400mg/kg) for 40 days, exhibited a significant reduction of food intake, body weight, lee index, serum lipids, atherogenic index, coronary risk index and inversely increased the level of brain serotonin in rats. Thus, the present study indicates that *Hiptage madablota* root extract possessed significant anti-obese efficacy due to its hypophagic and hypolipidemic effects and provoke the brain serotonin level in rats fed on high fat diet.

Keywords: *Hiptage madablota,* Anti-obesity, Lee index, Serotonin, High fat diet.

1. Introduction

Nowadays, obesity has become a global health problem because it is accompanied with various metabolic syndromes such as arteriosclerotic disease, type 2 diabetes mellitus and fatty liver disease (Després and Lemieux, 2006). In obese-diabetic patients, the pancreas loses its function of releasing insulin and insulin resistance is developed due to the excessive deposition of fats in non adipose tissue, leading to either cell death or cell dysfunction (Semenkovich, 2006; Olefsky, 2008). Despite the management or control of obesity, anti-obesity drugs are limited and they prevent the fat absorption by inhibiting lipid breakdown in intestine (orlistat) or reducing the appetite by increasing the satiety and modulating the central nervous system (sibutramine and rimonabant) (Choi *et al*., 2007). Moreover, these drugs produce severe cardiac and psychiatric side effects. Therefore, in recent years, there has been a great increase in the use of herbal drugs for the treatment of obesity (Shi and Burn, 2004).

Hiptage madablota Gaertn. (Malpighiaceae), native from India to the Philippines, is a vine like plant that is often cultivated in the tropics for its attractive and fragrant flowers. It can be trimmed to form a small tree or shrub or can be trained as a vine (Whistler, 2000). *Hiptage madablota (H. madablota)* root is cultivated in India for medicinal purposes (Bailey and Bailey, 1976). Traditionally, the root of *H. madablota* used in the treatment of obesity and reduced the weight gain. The folklore's claim of the utility of this plant in treatment of obesity has not been scientifically evaluated. For this reason, the present study attempts to explore the effect of *H.madablota* root on energy balance disorders like obesity, hyperphagia, and hyperlipidemia. Therefore, the present study is carried out to investigate the anti-obesity efficacy of the ethanol extract of root of *H.madablota* (EEHM) in high fat diet induced obesity rats.

2. Material and Methods

2.1. Collection of Plant Material and Preparation of Extract

The roots of *H. madablota* were collected from Tirumala hills, Chittoor district of Andhra Pradesh, India in March 2011. The plant was authenticated by Dr. K. Madhava Chetty, Assistant Professor, Department of Botany, Sri Venkateswara University, Tirupati. The root of *H. madablota* was dried in shade and pulverized in the grinder-mixer to obtain a coarse powder, which was passed through the 60 mesh sieve. A weighed quantity (100g) of powder was subjected to continuous hot extraction with ethanol in soxhlet apparatus for 48

* Corresponding author. e-mail: saaradhana007@gmail.com.

hours. Then the extract was evaporated at reduced pressure using rotary evaporator until all the solvent has been removed to give an extract sample.

2.2. Preliminary Phytochemical Investigation of EEHM

The preliminary phytochemical investigation was performed using standard phytochemical tests for the presence of various phytoconstituents in the EEHM (Harbone, 1998).

2.3. Quantification of Total Saponins in EEHM

Total saponins were quantified in EEHM by Gravimetric method. Accurately weighed 2g of the EEHM in a beaker, added 150 ml ethanol and boiled on a water bath for 10-15 minutes. The ethanol layer filtered to another beaker. Extracted the precipitate with 100 ml of ethanol and boiled on water bath for 10-15 minutes. Above process is repeated twice (with 75 ml of ethanol). The filtrate were combined and concentrated to about 20ml. To this, 150 ml of dry acetone was added to precipitate the saponins. It was filtered and dried at 100°C for constant weight (Hudson and El-Difrawi, 1979). Percentage of total saponins is calculated by (Weight of residue/ Weight of sample taken)X100

2.4. Animals

Female albino Wistar rats (150-180g) were obtained from the animal house in Sree Vidyanikethan College of Pharmacy, Tirupati, Andhra Pradesh. Female albino Wistar rats (150-180g) were obtained from the animal house in Sree Vidyanikethan College of Pharmacy, Tirupati, Andhra Pradesh. The animals were maintained in a well-ventilated room with 12:12 hour light/dark cycle in polypropylene cages and fed with standard pellet diet (Hindustan lever limited, Bangalore) and water was given ad libitum. Animals were maintained under standard housing conditions (room temperature 24–27◦C and humidity (60–65%). The experiments were performed after approval (Approval no: SVCP/IAEC/I-026/2011-12) of the protocol by the Institutional Animal Ethics Committee (IAEC) and were carried out in accordance with the current guidelines on the care of laboratory animals.

2.5. Acute Oral Toxicity Studies

Acute oral toxicity studies of EEHM were done as per the OECD guideline no. 423 (Acute toxic class method). Albino wistar rats (n=6) either sex selected and kept fasting for overnight providing only water. EEHM was administered orally at the dose level of 2000mg/kg by oral needle and observed for 14 days. The animals were observed for gross behavioral, neurological, autonomic and toxic effects at short intervals of time for 24 h and then daily for 14 days (OECD, 2002).

2.6. Induction of Experimental Obesity

The rodent feed was mixed to following ingredients to prepare high fat diet (HFD): Casein-20%, D, L methionine-0.3%, corn starch-15%, sucrose-27.5%, cellulose powder-5%, mineral mixture-3.5%, vitamin mixture-1%, choline bitartrate-0.2%,corn oil -9.9%, lard oil-17.6% (Vasselli *et al.,* 2005). The high fat diet was prepared, dried, powdered and administered every day in morning to animals with water ad libitum. Weight gain was observed in rats on third day, therefore, confirming the development of obesity in rats. This diet was continued for 40 days.

2.7. Anti-Obesity Efficacy of EEHM in HFD- Fed Rats

Thirty six female Wistar rats (150-180g) were randomly divided into six groups of six animals each. The following schedule of dose, diet administration in experimental groups was followed: Group I animals received vehicle 0.2ml of 1% CMC and were considered as normal control; Group II animals received high fat diet + Vehicle 0.2ml of 1% CMC; Group III, IV and V animals received high fat diet + EEHM of 100, 200 and 400 mg/kg body weight, per oral, respectively; Group VI animals received high fat diet + Orlistat 50 mg/kg body weight, per oral for 40 days. The body rectal temperature was measured on day 41 in order to estimate thermogenesis. The food intake of each animal was measured every day. Body weight of animal was recorded to assess the weight gain (Vasselli *et al*., 2005).

2.8. Determination of Adiposity Level in Normal and HFD- Fed Rats

The Lee index is used for measuring the adiposity level of animals because it is highly correlated with the percentage of the total body fat (Lee, 1929; Li *et al*., 1998). The Lee's index is expressed as cubic root of body weight in grams divided by the naso-anal length in millimeters multiplied by 10^4.

2.9. Estimation of Lipid Profile in Normal and HFD-Fed Rats

On day 41, the animals were sacrificed by cervical dislocation and the whole brain was dissected out for estimation of serotonin. Blood samples were collected by cardiac puncture and allowed to stand for 30 min at 37°C. The clear serum was separated at 2500 rpm for 10 min and was used for the estimation of Serum total cholesterol (TC), triglycerides (TG), HDL-C were measured with the help of commercially available diagnostic kits (Span Diagnostics, India). The serum low-density lipoprotein (LDL-C), very low-density lipoprotein (VLDL-C) levels, the atherogenic index and coronary risk index were calculated by Friedewald formula: VLDL-C = TG / 5; LDL-C = TC - (HDL-C + VLDL-C) (Friedwald *et al*., 1972). The atherogenic index and coronary risk index were calculated from LDL-C/ HDL-C and TC/ HDL-C respectively (Abbott *et al*., 1988; Alladi and Shanmugasundaram, 1989).

2.10. Estimation of Serotonin in Normal and HFD- Fed Rats

The striatum and hippocampus were separated from the brain and a standard procedure was followed for the estimation of serotonin by Spectrofluorimetry at 360-470 nm (Schlumpf *et al*., 1974).

2.11. Statistical Analysis

The present research observations were signified as Mean ± Standard Error Mean. Statistical significance of dissimilarities amid the groups was evaluated by one way and multiple way analysis of variance (ANOVA)

followed by Dunnett's test. *P* values less than 0.05 were deliberated as significance.

3. Results

3.1. Phytochemical analysis and Quantification of Total Saponins of EEHM

The percentage yield of EEHM was found to be 6.50%w/w. The preliminary phytochemical analysis of EEHM showed the presence of various phytochemical constituents like glycoside, flavonoids, terpenoids, saponins, phenolic compounds and tannins. By the Gravimetric method, it was found that 18.50% of total saponins were found in EEHM.

3.2. Acute oral toxicity of EEHM

Acute oral toxicity studies revealed that EEHM is safe up to 2000mg/kg. There was no lethality or toxic reactions such as tremors, convulsions, salivation, diarrhea, lethargy, sleep and coma.

3.3. Efficacy of EEHM on body temperature of normal and HFD- fed rats

Table 1 represents the body temperature of HFD-fed rats. The body temperature is significantly ($p<0.05$) increased after 0, 30, 60, 90, 180 min administration of EEHM when compared with group II animals' body temperature. EEHM (100 & 200 mg/kg bd.wt, per oral) treated groups exhibited ($p<0.05$) slight increases in body temperature. EEHM (400 mg/kg, b.wt, per oral) treated animals showed rise ($p<0.05$) in body temperature than standard drug orlistat (50 mg/kg bd.wt, per oral) treated animals.

3.4. Efficacy of EEHM on body weight and food intake of normal and HFD- fed rats

The body weight and food intake in the EEHM (100, 200 and 400 mg/kg bd.wt, per oral) were treated and vehicle treated groups were monitored for a 40-day treatment period. At the end of the study, the body weight and food intake of EEHM treated rats were significantly ($p<0.05$) lower than Group II animals. High doses of EEHM (400 mg/kg) significantly suppressed the weight gain and food intake in rats (Table 1).

3.5. Efficacy of EEHM on Lee's index of normal and HFD- fed rats

High fat diet caused a significant rise in Lee's index in group II rats. Orlistat (50 mg/kg) and EEHM (100, 200 and 400 mg/kg) treated rats showed a significant ($p<0.05$) dose dependent reduction in Lee's index when compared to group II animals.

3.6. Efficacy of EEHM on serum lipids of normal and HFD- fed rats

High fat diet induces significant elevations of serum total cholesterol (TC), triglycerides (TG), LDL-C, VLDL-C in untreated group II rats. Oral treatment with orlistat (50 mg/kg) and graded dose of EEHM (100, 200 and 400 mg/kg) caused a significant ($p<0.05$) reduction in the serum total cholesterol (TC), triglycerides (TG), LDL-C and VLDL-C of HFD- fed rats. Orlistat and EEHM (200 and 400 mg/kg) caused a significant improvement in HDL-C of HFD- fed rats (Table 3).

3.7. Efficacy of EEHM on Atherogenic and coronary risk index of normal and HFD- fed rats

Additionally, orlistat (50 mg/kg) and EEHM (100, 200 & 400 mg/kg) treated rats showed a significant ($p<0.05$) reduction in atherogenic and coronary risk index when compared to HFD induced obese rats (Table 3).

3.8. Efficacy of EEHM on serotonin level in normal and HFD- fed rats

HFD- fed rats had a reduced level of serotonin in the brain when compared to vehicle treated group rats (Group I). Treatment with EEHM (100, 200 and 400 mg/kg, per oral) and orlistat (50 mg/kg, per oral) restored ($p<0.01$) the decreased level of serotonin in the brain in a dose dependent manner (Figure 1).

Table 1. Efficacy of EEHM on body temperature of normal and HFD- fed rats

Grops	Treatment	Body temperature (min)					
		0	**30**	**60**	**90**	**120**	**180**
I	Vehicle 0.2ml of 1% CMC	36.59±0.1365	36.25±0.0322	36.05±0.1543	36.39±0.0821	36.28±0.0550	36.36±0.0877
II	High Fat Diet (HFD)	36.47 ±0.1156	36.58±0.0741	36.37±0.0684	36.50±0.0379	36.82±0.0310	37.27±0.0411
III	HFD + EEHM (100mg/kg, p.o)	36.33±0.0663	36.75±0.0471	37.23±0.0398*[b]	36.82±0.1828*[b]	37.26±0.0330*[b]	37.28±0.0358*[b]
IV	HFD + EEHM (200mg/kg, p.o)	36.46± 0.1137	36.92±0.0214*[b]	37.49±0.0612*[b]	37.39±0.0582*[b]	37.54±0.0254*[b]	37.47±0.0344*[b]
V	HFD + EEHM (400mg/kg, p.o)	36.48 ± 0.1141	37.06±0.1842*[b]	37.53±0.0989*[b]	37.37±0.0610*[b]	37.49±0.0618*[b]	37.71±0.0735*[b]
VI	HFD +Orlistat (50mg/kg, p.o)	36.44± 0.1108	37.61±0.0579*[b]	37.60±0.1103*[b]	37.55±0.0325*[b]	37.62±0.0878*[b]	37.56±0.0752*[b]

Values are mean ± SEM of six animals; Statistical significance test for comparisons was done by ANOVA, followed by Dunnett's test. Comparisons were made between: a) Group I vs Group II; b) Group II vs Group III, IV, V & VI; *p value < 0.05. p.o : per oral.

Table 2. Efficacy of EEHM on body weight, food intake& Lee Index of normal and HFD- fed rats

Parameters	Group I: Vehicle 0.2ml of 1% CMC	Group II: High Fat Diet control	Group III: HFD + EEHM (100mg/kg,p.o)	Group IV: HFD + EEHM (200mg/kg, p.o)	Group V: HFD + EEHM (400mg/kg,p.o)	Group VI: HFD + Orlistat (50mg/kg,p.o)
Initial body Weight (g/rat)	186.83±1.939	188.5±2.335	189.17±1.641	187.00±2.129	189.33±1.833	189.17±2.167
Body Weight on 40th day (g/rat)	212.50±1.928*^{a}	255.33±4.499	241.67±1.202*^{b}	229.33±2.578*^{b}	221.50±1.708*^{b}	210.67±3.283*^{b}
Weight gain (g/rat)	25.67±1.585*^{a}	66.83±2.926	52.50±1.784*^{b}	42.33±1.585*^{b}	31.67±0.9545*^{b}	23.33±1.116*^{b}
Food intake (g/rat/day)	21.67±0.6667*^{a}	31.50±0.7638	25.83±0.6009*^{b}	23.67±0.4944*^{b}	22.33±0.7601*^{b}	20.50±1.118*^{b}
Lee Index	244.34±2.359*^{a}	311.12±4.632	276.97±1.577*^{b}	252.12±0.9481*^{b}	245.98±2.033*^{b}	240.05±1.646*^{b}

Statistical significance test for comparisons was done by ANOVA, followed by Dunnett's test; Comparisons were made between: a) Group I vs Group II; b) Group II vs Group III, IV, V & VI; *p value < 0.05. p.o : per oral.

Table 3. Efficacy of EEHM on Serum lipids, Atherogenic Index & Coronary Risk Index of normal and HFD- fed rats

Parameters	Group I: Vehicle 0.2ml of 1% CMC	Group II: High Fat Diet control	Group III: HFD + EEHM (100mg/kg, p.o)	Group IV: HFD + EEHM (200mg/kg, p.o)	Group V: HFD + EEHM (400mg/kg, p.o)	Group VI: HFD + Orlistat (50mg/kg, p.o)
TC (mg/dl)	114.33±1.430*^{a}	231.50±3.085	160.33±2.376*^{b}	139.17±2.522*^{b}	121.67±3.085*^{b}	117.67±2.860*^{b}
TG (mg/dl)	113.17±1.721*^{a}	172.16±2.868	144.33±2.539*^{b}	133.67±1.944*^{b}	121.67±0.8819*^{b}	114.00±1.291*^{b}
LDL (mg/dl)	38.53±1.155*^{a}	164.73±3.838	93.80±2.309*^{b}	70.10±1.578**^{b}	53.83±2.735*^{b}	45.20±2.686*^{b}
VLDL (mg/dl)	22.63±0.3442*^{a}	34.43±0.5737	28.87±0.5077*^{b}	26.73±0.3887*^{b}	24.33±0.1764*^{b}	22.63±0.3844*^{b}
HDL (mg/dl)	53.17±1.352*^{a}	32.33±1.358	37.67±1.358*^{b}	42.33±1.022*^{b}	43.50±1.022*^{b}	49.67±1.282*^{b}
Atherogenic Index (AI)	0.73±0.0339*^{a}	5.15±0.2871	2.51±0.1246*^{b}	1.66±0.0372*^{b}	1.24±0.0662*^{b}	0.91±0.0580*^{b}
Coronary Risk Index (CRI)	2.15±0.040*^{a}	7.23±0.3298	4.28±0.1484*^{b}	3.29±0.0468*^{b}	2.80±0.0715*^{b}	2.37±0.0635*^{b}

TC - Total Cholesterol; TG - Triglycerides; VLDL – Very Low Density Lipoproteins; HDL - High Density Lipoproteins

Values are mean ± SEM of six animals; Statistical significance test for comparisons was done by ANOVA, followed by Dunnett's test. Comparisons were made between: a) Group I vs Group II; b) Group II vs Group III, IV, V & VI; *p value < 0.05. p.o : per oral.

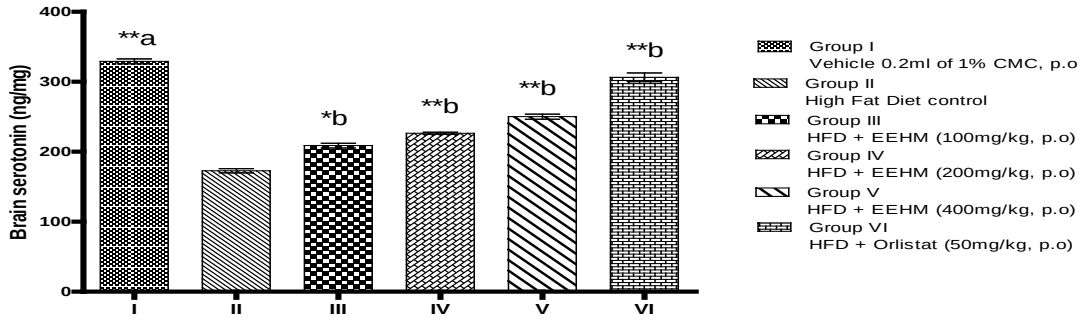

Figure 1. Efficacy of EEHM on Brain serotonin Level of normal and HFD- fed rats

Values are mean ± SEM of six animals; Statistical significance test for comparisons was done by ANOVA, followed by Dunnett's test. Comparisons were made between: a) Group I vs Group II; b) Group II vs Group III, IV, V & VI; *p value < 0.05; **p value < 0.01. p.o : per oral.

4. Discussion and Conclusion

Obesity is a metabolic disorder which occurs due to an energy imbalance between an increased ratio of caloric intake and a decreased ratio of energy expenditure. Obesity is associated with disorders of hyperlipidemia, atherosclerosis and diabetes mellitus. In modern medicine, there is a great increase in the use of evidence-based complementary treatments like natural remedies in the management and prevention of obesity (Das and Maulik, 2006; Sharpe *et al*., 2007).

In the present study, the anti-obesity efficiency of ethanolic extract of root of *H.madablota* is evaluated at 100,200 and 400mg/kg in normal and HFD-fed rats. Acute oral toxicity studies revealed that ethanol extract of *H.madablota* is found to be safe up to the dose of 2000mg/kg, p.o.

Body temperature of the HFD-fed rats was significantly (p<0.01, p<0.05) increased after 0, 30, 60, 90, 180 min administration of EEHM when compared with group II. EEHM has increased body temperature by overall stimulant & thermogenesis property because obesity is associated with defective thermogenesis. These mechanisms confirmed with rodents and dogs, several β3-adrenoceptor agonists were shown to have potent thermogenic anti-obesity effects with classical adrenoceptor stimulation (Arch, 2008).

In case of body weight and adiposity level (lee index), animals which received extract suppressed the body weight gain and adiposity level (lee index) in a dose-dependent manner. Moreover, the previous studies (Haynes *et al.,* 2002; Arch, 1981) supported the body weight gain and adiposity level (lee index) was significantly decreased. In normal rats, the reference value of lee index is lower than 300. If the lee index values higher than 300, the rats classifies as obese (Bernardis and Patterson, 1968). EEHM significantly (p<0.01) decreased the Lee index when compared to HFD control.

EEHM reduced food intake by hypophagic activity because obesity is associated with hyperphagia. High fat diet caused significant elevations of serum total cholesterol (TC), triglycerides (TG), LDL-C, VLDL-C in untreated group II. Oral treatment with orlistat (50 mg/kg) and graded dose of EEHM caused a significant (p<0.01) reduction in the serum lipids on HFD- fed rats. EEHM may be attributed to lowering lipogenesis, enhancing lipolysis, suppressing appetite and decreasing lipid absorption (Shivaprasad *et al*., 2014).

The atherogenic and coronary artery risk indices were potent and authentic indicator for cholesterol deposition into tissues. In human, the normal reference range of atherogenic index and coronary artery risk index should not be exceeding above 4 and 2.5, respectively (Lee *et al.,* 2013; Murray and Pizzorno, 1998). The results of the present study showed that the atherogenic and coronary artery risk indices were significantly decreased in EEHM treated HFD-fed rats. Thus strongly confirmed that EEHM exhibit hypolipidemic effects.

HFD-fed rats had a reduced level of serotonin in brain when compared to vehicle treated group rats (Group I). Treatment with EEHM and orlistat (50 mg/kg, p.o) restored (p<0.01) the decreased level of the serotonin in the brain in a dose-dependent manner. Various in-vivo studies have contributed to the understanding of the anti-obesity effects via the regulation of the serotonin level. A typical reduction of food appetite and increased serotonin availability were observed in all the weight control studies on both animal and human subjects. These were associated with reduced levels of total cholesterol, LDL, triglycerides, and increased HDL level and urinary excretion of fat metabolites (Chuah *et al*., 2013).

Upon preliminary phytochemical studies of ethanolic extract revealed that the presence of glycoside, flavonoids, terpenoids, phenolic compounds and tannins and revealed that 18.5% saponins are present. Earlier studies reported that saponins and flavanoids mediate their hypophagic and hypolipidemic effects by reducing the food intake and inhibiting the lipid absorption or enhancing the enterohepatic excretion of cholesterol in the bile acid (Rajalakshmi *et al.,* 2004; Ruizc *et al*., 2005; Dixit *et al*., 2012).

In conclusion, oral administration EEHM, inhibit the food intake, weight gain, serum lipid profiles, atherogenic, coronary risk and Lee's index by hypophagic and hypolipidemic effects and elicit the brain serotonin level in HFD-fed rats. The presence of saponins is useful in the treatment of obesity and flavonoids, phenolic compounds & tannins have beneficial effects on potent hypolipidemic and anti-oxidant properties of EEHM. Further studies are needed not only to find the exact mechanism of action, but also to isolate and characterize the active phytocompounds for its effects.

Acknowledgement

The authors are grateful to Padmashree Dr. M. Mohan Babu, Chairman, Sree Vidyanikethan Educational Trust, Tirupati, India for providing the necessary facilities to carry out this work.

Declaration of Interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

References

Abbott RD, Wilson PW, Kannel WB and Castelli WP. 1988. High density lipoprotein-cholesterol, total cholesterol screening and myocardial infarction. The Framingham Study. *Arterosclerosis*, **8:** 207–211.

Alladi S and Shanmugasundaram KR. 1989.Induction of hypercholesterolaemia by supplementing soy protein with acetate generating amino-acids. *Nutrition Reports Inter*., **40:** 893–899.

Arch JR. 1981. The contribution of increased thermogenesis to the effect of anorectic drugs on body composition in mice. *Am J Clin Nutr*, **181**; 34:2763– 9.

Arch JR. 2008. The discovery of drugs for obesity, the metabolic effects of leptin and variable receptor pharmacology: perspectives from beta3-adrenoceptor agonists. *Naunyn Schmiedebergs Arch Pharmacol*, **378**: 225–240.

Bailey LH and Bailey EZ. 1976. **Hortus**.3rd ed. Macmillan General Reference, New York.

Bernardis LL and Patterson BD. 1968. Correlation between 'Lee index' and carcass fat content in weanling and adult female rats with hypothalamic lesions. *J Endocrinol*, **40:** 527- 528.

BulusuSitaram. 2006. Bhavaprakasa of Bhavamisra: Original Text Along With Commentary and Translation : Including Nighantu Portion – 2. Chaukhabha Orientalia (a house of oriental and antiquarian books), Vol I, pg 326.

Choi H, Eo H, Park K, Jin M, Park EJ, Kim SH, Park JE and Kim S. 2007. A water soluble extract from *Cucurbita moschata* shows anti-obesity effects by controlling lipid metabolism in a high-fat diet-induced obesity mouse model. *Biochem Biophys Res Commun*, **359:** 419–425.

Chuah LO, Ho WY, Beh BK and Yeap SK. 2013.Updates on Antiobesity Effect of Garcinia Origin (-)-HCA.*Evid Based Complement Alternat Med*, **2013**: 751658.

Das DK and Maulik N. 2006. Resveratrol in cardioprotection: a therapeutic promise of alternative medicine. *Molecular Interventions*, **6:** 36–47.

Després JP and Lemieux I. 2006.Abdominal obesity and metabolic syndrome. *Nature*, **444:** 881–887.

Dixit P, Prakash T, RoopaKarki and Kotresha D. 2012. Antiobesity effects of *Butea monosperma* (Lam) bark extract in experimentally induced obese rats. *Indian J Experimental Biol.*, **50**: 476-483.

Friedewald WT, Levy RI and Fredrickson DS. 1972. Estimation of VLDL and LDL-cholesterol in plasma, without use of the preparative ultracentrifuge. *Clin Chem*, **18:** 499–502.

Harbone JP. 1973. **Phytochemical Methods, A Guide to Modern Technique of Plant Analysis.** Chapmann and Hall, London, pp1-271.

Haynes AC, Chapman H, Taylor C, Moore GBT, Cawthorne MA, Tadayyon M, John C. Clapham JC and Arch JR. 2002. Anorectic, thermogenic and anti-obesity activity of a selective orexin-1 receptor antagonist in ob/ob mice. *Regulatory Peptides*, **104**: 153– 159.

Hudson BJF and El-Difrawi EA. 1979.The sapogenins of the seeds of four Lupin species. *J Plant Foods*, **3:**181-186.

Lee CL, Wen JY, Hsu YW and Pan TM. 2013. Monascus-Fermented yellow pigments monascin and ankaflavin showed antiobesity effect via the suppression of differentiation and lipogenesis inobese rats fed a high-fat diet. *J Agric Food Chem.*, **61:** 1493–1500.

Lee MO. 1929. Determination of the surface area of the white rat with its application to the expression of metabolic results. *Am J of Physiol.*, **89:**24–31.

Li H, Matheny M, Tumer N and Scarpace PJ. 1998. Aging and fasting regulation of leptin and hypothalamic neuropeptide Y gene expression. *Am J Physiol Endocrinol Metab.*, **275:** 405–411.

Murray MT and Pizzorno J. 1998.**Cholesterol.** In: Encyclopedia of Natural Medicine, 2nd edition, 347–400.

OECD. 2002. **Acute Oral Toxicity**. Acute oral toxic class method guideline 423 adopted in: Eleventh Addendum to the OECD, guidelines for the testing of chemicals organization for economical co-operation and development.

Olefsky JM. 2008. Fat talks, liver and muscle listen. *Cell*, **134:** 914–916.

Rajalakshmi D and Sharma DK. 2004. Hypolipidemic effect of different extracts of *clerodendron colebrookianum* walp in normal and high fat diet fed rats. *J Ethnopharmacol.*, **90:** 63-68.

Ruiz C, Falcocchio S, Xoxi E, Villo L, Nicolosi G, Pastor FIJ, Diaz P and Saso L. 2006. Inhibition of *Candida rugosa* lipase by saponins, flavonoids and alkaloids. *Journal of Molecular Catalysis B: Enzymatic*., **40:** 138–143.

Schlumpf M, Lichtensteiger W, Langemann H, Waser PG and Hefti F. 1974. A fluorimetric micromethod for the simultaneous determination of serotonin, noradrenaline and dopamine in milligram amounts of brain tissue. *BiochemPharmacol.*,**23:** 2337-446.

Semenkovich CF. 2006. Insulin resistance and atherosclerosis. *J Clin Invest.,* **116:** 1813–1822.

Shalaby M A and Hammouda A A-E. 2013. Antiobesity, antioxidant and antidiabetic activities of red Ginseng plant extract in obese diabetic rats. *J Intercult Ethnopharmacol.*, **2(3)**: 165-172.

Sharpe PA, Blanck HM, Williams JE, Ainsworth BE and Conway JM. 2007. Use of complementary and alternative medicine for weight control in the United States. *J Altern Complementary Med.*, **13:** 217–222.

Shi Y and Burn P. 2004. Lipid metabolic enzymes: emerging drug targets for the treatment of obesity. *Nat Rev Drug Discovery*, **3:** 695–710.

Shivaprasad H N, Gopalakrishna S, Mariyanna B, Thekkoot M, Reddy R and Tippeswamy B S. 2014. Effect of *Coleus forskohlii* extract on cafeteria diet-induced obesity in rats. *Pharmacog Res*, **69**: 41-45.

Vasselli JR, Weindruch R, Heymsfield SB, Pi-Sunyer FX, Boozer CN, Yi N, Wang C, Pietrobelli A and Allison DB. 2005. Intentional weight loss reduced mortality rate in a rodent model of dietary obesity. *Obes Res.*, **13:** 693-702.

Whistler WA. 2000. **Tropical Ornamentals**. Timber Press, Portland.

JJBS

Volume 7, Number 2, June .2014
ISSN 1995-6673
Pages 119 - 126

Jordan Journal of Biological Sciences

Intakes of Fats, Cholesterol, Fiber and Micronutrients as Risk Factors for Cardiovascular Disease in Jordan

Hamed R. Takruri[1,*] and Refat A. Alkurd[2]

[1]Department of Nutrition and Food Technology, Faculty of Agriculture, The University of Jordan

[2]Department of Nutrition, Faculty of Pharmacy and Medical Sciences, University of Petra, Jordan

Received: December 22, 2013 Revised: February 6, 2014 Accepted: February 14, 2014

Abstract

The cardiovascular disease (CVD) is the leading cause of death in Jordan and many other countries. The etiology of CVD is multifactorial. Dietary factors play significant roles in the initiation, prevention and treatment of CVD diseases. The aim of this study is to estimate the daily intake of nutrients associated with developing CVD among Jordanians, based on the Department of Statistics household budget survey "JHEIS 2010", and to detect any changes in these intakes in comparison with the previous JHEIS survey. The data of the JHEIS 2010 were analyzed for the purpose of estimating the quantity of nutrient intakes in the different governorates of the country. The results showed that energy intake in the whole country (the Kingdom) was 3325 kcal/day. The daily intakes (as % of energy) of total, saturated, polyunsaturated, monounsaturated, trans, omega-3 and omega-6 fats were 26.6, 6.5, 8.5, 8, 0.2, 0.21 and 2.2%, respectively. The daily fiber intake was 7.2 g/1000 kcal. The daily intakes (mg/day) of cholesterol, sodium, potassium, calcium, and magnesium were 303, 6206, 3030, 627 and 305, respectively. There was a variation in the intakes of these nutrients and energy among governorates. It is concluded that the Jordanian estimated daily intakes of total fat, saturated, polyunsaturated and trans fats were within the recommendations expressed as percent of energy intake, in contrary to the intakes of monounsaturated, omega-3 and omega-6 fats and dietary fibers, which were lower than those of the recommendations. In addition, the daily intakes of potassium, calcium and magnesium were low and those of energy and sodium intakes were very high as compared with the recommendations.

Keywords: Jordan, Trans Fatty Acids, Saturated Fatty Acids, Cholesterol, Cardiovascular Disease, Hypertension, Sodium, Potassium, JHEIS 2010.

1. Introduction

The cardiovascular disease (CVD) is a major cause of disability and premature death throughout the world (WHO, 2007). Atherosclerotic risk factors, such as hypertension, smoking and alcohol, represent significant predictors for several CVDs (US Census Bureau, 2004a,b). In addition, there is a dramatic increase in the conditions that trigger heart disease and other chronic illnesses (Musaiger and Al-Hazzaa, 2012), particularly in low- and middle-income countries (WHO, 2012). Few studies have investigated the risk factors associated with lifestyle and family history of hypertension in Arab populations.

The health and nutritional status in the Arab Middle East countries has changed during the past four decades as a result of changes in dietary habits, socioeconomic situation and lifestyle. Coronary heart disease (CHD), diabetes, hypertension and cancer have become the main health problems in these countries (Fahed *et al.*, 2012; Musaiger, 2012). The burden of these non-communicable diseases is associated with an increased risk of CVD and increased health care costs (Brown *et al.*, 2009). The estimated mortality rate due to CVD and diabetes in the Eastern Mediterranean Region ranged from 179.8 to 765.2 per 100,000 population, with the highest rates in poor countries. Also, the prevalence of overweight and obesity (body mass index ≥25 kg/m2) has reached an alarming level in most countries of the region, ranging from 25% to 82%, with a higher prevalence among women (Musaiger and Hazzaa, 2012).

In Jordan, major causes of death and disability have shifted from nutritional deficiencies and infectious diseases to chronic diseases such as CVD, cancers and diabetes (Stovall *et. al.*, 2013; Madanat *et. al*., 2008). Therefore, the non-communicable diseases and obesity have become a public health concern. Al-Nsour *et al*. (2012) reported that 30% of adult Jordanians, older than 18 years of age, were overweight and 36% were obese. Hypertension was also found to be highly prevalent in Jordan, with 20.6% of the population reported to suffer from it (US Census Bureau, 2004a,b).

Jordan, like many other neighboring countries, has undergone a nutrition transition characterized by increased intakes of modern diets high in fat, cholesterol,

* Corresponding author. e-mail: htakruri@ju.edu.jo.

sugar and salt, and by decreased intakes of the traditional diets that are high in fiber, whole grains, fruits and vegetables (Madanat *et al.*, 2008; Musaiger, 2002; Popkin, 2002). Some of the effects of the nutrition transition are the increasing incidence of obesity, diabetes and CVD such as atherosclerosis, hypertension and stroke (Popkin, 2002).

CVD is associated with the "Western" diet style, which characterized by a high consumption of energy, fat, cholesterol, sugar and salt. CVD mortality rates are twice as high among segments of societies that follow such a diet than among people who eat sensibly (Scarborough *et al*., 2011; Hu, 2008). Salt, sugar, saturated fat and trans fats are harmful when consumed in excess; conversely, fruits and vegetables (which are rich in potassium, antioxidants and fiber), polyunsaturated fats, monounsaturated fats, whole grains, pulses, nuts and fish have consistently shown a protective effect against CVD (Scarborough *et al*., 2011; Hu, 2008).

Of all dietary lipids, saturated fat has the strongest effect on blood cholesterol levels. A high intake of saturated fat, particularly when combined with a low intake of polyunsaturated fat, will lead to increased low density lipoprotein (LDL) (bad cholesterol) (Elmadfa and Kornsteiner, 2009). Replacing saturated fat with monounsaturated and polyunsaturated fats can generally lower LDL levels (Rolfes *et al*., 2012).

Trans fats are toxic; by raising serum LDL and reducing high density lipoprotein (HDL), they substantially increase the risk of CHD and stroke (Mozaffarian and Stampfer, 2010). The US Department of Agriculture Dietary Guidelines for Americans (USDA, 2010) recommends limiting intake of trans fatty acids and saturated fatty acids (SFAs) to as little as possible. Similarly, the UK Government recommends consuming <2% of total energy in the form of these fats (Mozaffarian and Stampfer, 2010).

Epidemiological and clinical studies suggest that the intake of omega-3 (n-3) fatty acids contributes to the reduction of cardiovascular mortality and has beneficial effects on lipid profile as well as blood pressure (Cabo *et al.*, 2012), whereas an imbalance between dietary n-3 and omega-6 (n-6) fatty acids contributes to a wide range of diseases (Wertz, 2009). It is noteworthy that the optimum n-6/n-3 ratio has been estimated to be within the range of 5:1 to 10:1 (Jones and Kubow, 2006). Since the typical Western diet is rich in linoleic acid (LA) (and arachidonic acid (AA), and relatively poor in EPA/DHA, it has been proposed that lowering the dietary n–6/n–3 ratio would provide an efficient means by which to reduce the risk of CVD (Simopoulos, 2008).

Results from many studies suggest an inverse association between dietary fiber intake and CHD risk in the general population (Erkkilä and Lichtenstein, 2006). According to the American Heart Association (AHA), Diet Recommendations for Cardiovascular Disease Risk Reduction, Therapeutic Lifestyle Change (TLC), and Dietary Approach to Stop Hypertension (DASH) dietary patterns emphasize the importance of a higher consumption of fruit, vegetables, legumes, and whole grains, as they contain adequate fiber to lower the LDL cholesterol (Raymond and Couch, 2012). In addition, many other factors have been incriminated in hypertension pathogenesis, including changes in intracellular concentrations of calcium, sodium, potassium, and magnesium. Observational studies have shown that a diet rich in potassium, magnesium, and calcium, present mainly in fruits and vegetables, is associated with lower incidence and mortality from CVD (He *et al.*, 2006).

The present average sodium intakes, approximately 3000 to 4500 mg/day in various industrialized populations, are very high, that is, 2- to 3-fold in comparison with the current DRI of 1500 mg. By contrast, the present average potassium, calcium, and magnesium intakes are remarkably lower than the DRI (Lichtenstein *et al.*, 2006). There is convincing evidence indicating that this imbalance, between the intake of sodium, on the one hand, and the intakes of potassium, calcium, and magnesium, on the other, produce and maintain elevated blood pressure in a big proportion of the population. Thus, it was suggested that decreasing the intake of sodium and increasing the intakes of potassium, calcium, and magnesium would help in its prevention and basic treatment (Cunha *et al*., 2012; Lichtenstein *et al*., 2006). The purpose of this study is therefore to estimate the daily intakes of nutrients associated with developing CVD among Jordanians, based on the Department of Statistics household budget survey "JHEIS 2010", and to detect any changes in these intakes in comparison with the previous JHEIS survey.

2. Methods

Data in this paper were based on a household budget survey, namely the Jordanian Household Expenditures and Income Survey (JHEIS), 2010, which aimed to collect detailed data on the household expenditures and income. The raw data collection of this survey was conducted during the period of April 1, 2010 to April 30, 2011 (DOS, 2012).

The annual per capita food intake data of a representative sample of all Jordanian households were calculated. The included 13866 households were proportionally distributed among the different governorates of the whole country (Kingdom) using two-stage cluster stratified sampling method. These households included 73490 individuals, by an average of 5.3 capita/ household. Males constituted 51.26% of the sample, 40.2% were children younger than 18 years and 3.8% were elderly (older than 65 years). Each participating household received a questionnaire that contained data of expenditure on different food and nonfood categories. The data on food items were analyzed using a nutrition analysis software program (Food Processor SQL nutrition and fitness software, 2008) which included details on the contents of energy and nutrients for each food item. In case a food item was not included in the database of the mentioned program, the nutrient makeup of this food was obtained from other food analysis sources such as Food Composition Tables for Use in the Middle East (Pellet and Shadarevian, 1970) and Food Composition Tables of the Gulf Region (Musaiger, 2006). Such foods and their analyses were

introduced to the Food Processor database. Then the daily intakes of total fats, n-3 and n-6 fatty acids, saturated, monounsaturated, polyunsaturated and trans fatty acids, cholesterol, dietary fibers, sodium, calcium, phosphorus and magnesium were calculated. The nutrient intake values, obtained for the different governorates and the Kingdom, were compared with the highest daily requirement intake (DRI) and other professional health recommendations to assure that needs were met for all age groups (IOM, 2010; 2002/2005).

To compare the estimated energy intake of the Kingdom of Jordan with a reference value, the estimated energy requirements (EER) values for low active individuals were calculated and used for the purpose of comparison. The EER values were, respectively, 2680 and 2105 kcal for the reference man and woman of the age 25 years (IOM, 2002/2005; IOM, 2001).

The DRI's of some minerals and n-3 and n-6 fatty acids for the age groups of 19-30 and 31-50 years were used for comparison (IOM, 2010; 2002/2005). The Acceptable Macronutrient Distribution Range (AMDR) for >19 years old, as a percent of total energy intake, were also used for comparison (IOM, 2002/2005).

3. Results and Discussion

The kingdom (whole country) per capita energy consumption was found to be 3325 kcal/day (Table 1). The daily energy per capita intake of the governorates ranged from 2753 kcal/day in Ajloun to 4334 kcal/day in Madaba. The present daily energy intake of the Kingdom was 24% and 56% higher than that of the EER reference man and woman, respectively. Also when compared with the daily energy intake obtained in a 2006/2007 survey, the present daily energy intake was 9.7% higher (Takruri *et. al*., 2011). It was found that the present daily energy intake of 8 out of the 12 governorates was higher than that obtained in the previous survey, ranging from 3% (in Aqaba) to 40.5% (in Jerash), whereas 3 governorates had a lower daily energy intake than that of the previous survey; only Ma'an had the same daily energy intake reported in the previous survey (DOS, 2008; Alkurd, 2011). On the long run, if this high daily energy consumption pattern continues, it is expected to cause overweight and obesity with the association of increased high body fat. Overweight and obesity are two of the main risk factors of CVD (Musaiger and Al-Hazzaa, 2012).

The share of the total daily fat intake as a percent of daily energy intake in the Kingdom was 27.1% (Tables 2 and 3).

The contribution of fat to total daily energy intake ranged from 17.8% (in Mafraq) to 29.3% (in Amman). All these percentages (except in Mafraq) fall within the range of the Acceptable Macronutrient Distribution Range (AMDR) of fat (20%-35%) (IOM, 2002/2005). This indicates that the Jordanian consumption of fat as a percentage of the total daily energy intake is generally acceptable.

Table 1. Estimated daily *per capita* intake of energy (kcal) in the whole country and governorates in Jordan: comparison between JHEIS 2010 and JHEIS 2006/2007[1]

Governorate	2010	2006/2007	% of change
Amman	3327	2940	+ 13.2
Balqa	2921	3079	- 5.1
Zarqa	3272	2893	+ 13.1
Madaba	4334	3328	+30.2
Irbid	3143	3320	- 5.3
Mafraq	3285	2876	+14.2
Jarash	4290	3054	+40.5
Ajloun	2753	3245	-15.2
Karak	3925	3107	+26.3
Tafilah	3235	2710	+19.4
Ma'an	3074	3075	0.0
Aqaba	2833	2750	+3.0
Whole country	3325	3031	+9.7

[1]References: DOS, 2012; DOS, 2008; Alkurd,2011

It has been shown that the daily per capita fat supplies showed an impressive increase in most of the Middle East Arab countries, ranging from 13.6% in Sudan to 143.3% in Saudi Arabia (Musaiger, 2002). Despite these apparently acceptable percentages, the total amounts of fat consumed are high when attributed to the reference EER. When these amounts are attributed to the 2680 kcal/day, the share of the Kingdom as a percentage of the total estimated daily energy intake was 33.6%, whereas Ma'an, with the highest governorate daily intake, attained 60.4% and Mafraq, with the lowest daily intake, attained 22% of the EER. It is clear that when the total daily energy intake is higher than the required, the amount of the consumed fat is more critical to health than its percentage of the total energy consumed per day. The present total fat daily intake of the Kingdom in the present survey is 14.9% higher than that of the 2006/2007 survey (Alkurd, 2011) (see Table 2). Compared to the previous survey, the change in the total fat for the governorates ranged from +127% (in Ma'an) to -35% (in Ajloun). It is worth noting that the daily energy intake of Ma'an was not different, whereas its fat daily intake was increased by 127% (from 79.4 to 180.0); this indicates that the increase in the daily fat intake was at the expense of carbohydrate and protein.

As indicated in Tables 2 and 3, the Kingdom's SFA daily intake was 24 g, which is 6.5% of the estimated total daily energy intake. The 2006/2007 percentage was 6.2 (Alkurd, 2011), which is slightly lower than the present daily intake. Despite that, the present percentage of SFA intake is within the healthy recommendations of <10% of the total energy intake.

Table 2. Daily *per capita* estimated intake of different fats in the whole country and governorates in Jordan based on JHEIS 2010 and JHEIS 2006/2007[1]

Governorate	Daily intake of fat types (g)													
	Total fat		SFA		MUFA		PUFA		Trans		n-3		n-6	
	2010	2006/2007	2010	2006/2007	2010	2006/2007	2010	2006/2007	2010	2006/2007	2010	2006/2007	2010	2006/2007
Amman	109.9	92.5	26.9	22.7	36.6	28.1	31.7	28.5	0.62	0.91	0.82	0.77	8.66	7.8
Balqa	70.9	89.4	17.7	22.2	20.2	23.8	21.9	28.9	0.63	0.80	0.66	0.59	7.02	6.8
Zarqa	102.9	85.3	25.6	20.8	34.0	25.4	29.7	26.7	1.03	1.08	0.75	0.65	8.16	6.9
Madaba	110.3	97.3	22.9	21.3	30.9	28.2	39.8	33.7	0.55	1.13	0.83	0.75	9.29	8.2
Irbid	97.0	111.4	23.6	26.3	30.2	37.3	27.5	32.2	0.82	1.32	0.78	0.84	7.92	9.2
Mafraq	65.4	79.2	17.9	20.2	21.5	25.6	13.8	22.9	0.70	1.81	0.71	0.59	7.49	7.4
Jarash	126.9	82.1	29.2	21.9	36.7	23.4	37.3	23.2	1.66	1.03	0.94	0.67	10.07	7.7
Ajloun	73.4	112.9	19.0	25.0	20.7	38.2	20.0	37.6	1.35	1.89	0.61	0.86	6.72	8.9
Karak	107.3	74.1	23.5	16.8	32.1	22.7	34.5	25.0	0.73	1.16	0.75	0.66	8.88	7.7
Tafilah	88.7	64.9	27.5	15.0	23.0	19.6	23.3	20.3	0.48	0.95	0.66	0.60	8.34	6.7
Ma'an	180.0	79.4	22.8	18.0	30.0	27.1	17.9	23. 5	0.32	1.97	0.40	0.57	4.81	7.3
Aqaba	85.3	75.3	19.9	21.7	28.4	26.1	25.2	18.5	0.59	0.89	0.55	0.61	6.45	7.0
Whole country	100.0	87.0	24.0	21.0	31.6	27.1	29.7	27.0	0.72	1.25	0.77	0.68	8.18	7.6
% of change (Kingdom from 2006/2007 to 2010)	+14.9		+14.3		+16.6		+10		-42.4		+13.2		+7.6	

[1]References: DOS, 2012; DOS, 2008; Alkurd, 2011

Table 3. The percentage share of fats out of total energy intake (TEE) in the whole country and governorates in Jordan based on JHEIS 2010 and JHEIS 2006/2007[1]

Governorate	fats % share of TEE													
	Total fat		SFA		MUFA		PUFA		*Trans*		n-3		n-6	
	2010	2006/2007	2010	2006/2007	2010	2006/2007	2010	2006/2007	2010	2006/2007	2010	2006/2007	2010	2006/2007
Amman	29.3	28.3	7.3	6.9	9.9	8.6	8.6	8.7	0.2	0.3	0.22	0.24	2.3	2.4
Balqa	21.5	25.9	5.4	6.4	6.2	6.9	6.8	8.4	0.2	0.2	0.20	0.17	2.2	2.0
Zarqa	27.5	26.4	7.1	6.4	9.4	7.9	8.2	8.3	0.3	0.3	0.21	0.20	2.2	2.1
Madaba	22.4	26.2	4.8	5.7	6.4	7.6	8.3	9.1	0.1	0.3	0.17	0.20	1.9	2.2
Irbid	27.4	30.0	6.8	7.1	8.7	10.1	7.9	8.7	0.2	0.4	0.22	0.23	2.3	2.5
Mafraq	17.8	24.7	4.9	6.3	5.9	8.0	3.8	7.2	0.2	0.6	0.19	0.18	2.1	2.3
Jarash	26.2	24.0	6.1	6.4	7.7	6.8	7.8	6.8	0.4	0.3	0.20	0.20	2.1	2.3
Ajloun	23.7	31.0	6.2	6.9	6.8	10.5	6.5	10.4	0.4	0.5	0.20	0.24	2.2	2.5
Karak	24.1	21.4	5.4	4.9	7.4	6.6	7.9	7.2	0.2	0.3	0.17	0.19	2.0	2.2
Tafilah	24.4	21.5	7.6	5.0	6.4	6.5	6.5	6.7	0.1	0.3	0.18	0.20	2.3	2.2
Ma'an	24.4	23.1	6.7	5.2	8.8	7.9	5.2	6.8	0.1	0.6	0.12	0.17	1.4	2.1
Aqaba	26.7	24.6	6.3	7.1	9.0	8.5	8.0	6.0	0.2	0.3	0.17	0.20	2.1	2.3
Whole country	**27.1**	25.6	6.5	6.2	8.5	8.0	8.0	7.9	0.2	0.4	0.21	0.20	2.2	2.3
% of change (Kingdom 2006/2007 to 2010)	+5.9		+4.8		+6.3		+1.3		-50		+5		-4.5	

[1]References: DOS 2012; DOS 2008; Alkurd, 2011

When these amounts are attributed to the EER of 2680 kcal, the Kingdom SFA intake constitutes 8% of this EER which is still within the recommendations for healthy people. The consumed saturated fat percentages of the governorates ranged from 4.8% in Madaba to 7.6% in Tafilah. Compared with the American Heart Association 2006 diet recommendations for CVD risk reduction that recommend limiting the daily intake of saturated fat to <7% of the total daily energy intake; it is clear that the present daily intake is higher than that mentioned in this recommendation (Lichtenstein *et. al*., 2006).

The Kingdom's daily intake of MUFA was 31.6 g which is 8.5% of the total daily energy intake. When this daily intake is referred to the 2680 kcal, it constitutes 10.6% of this energy, which is at the lower level of the healthy recommendations. Compared to the percentage obtained in the 2006/2007 survey, which was 8% (Alkurd, 2011), the present daily intake is slightly higher, but is still lower than the recommendations of 10-15% (Raymond and Couch, 2012).

The Kingdom's daily intake of PUFA was 29.7 g which is 8% of the total daily energy intake. The present percentage is similar to the percentage of the 2006/2007 survey of 7.9% (Alkurd, 2011). The present percentage is within the healthy recommendations (≤10%) of total daily energy intake.

Regarding the *trans* fats daily intake, it was found to be 0.7 g for the Kingdom, which is 0.2% of the total daily energy consumption. The percentages of daily intake of all governorates is <0.5. It's clear that these percentages are very low. The healthy recommendations of *trans* fats state that the daily intake should be as low as possible while consuming a nutritionally adequate diet (USDA, 2002/2005). The American Heart Association 2006 diet recommendations for CVD risk reduction state that the healthy daily intake should be <1% of the total daily energy intake; the present daily intake is consistent with this recommendation (Lichtenstein, 2006). The present percentage of *trans* fats is half that of the 2006/2007 daily intake (Alkurd, 2011). This is probably due to the relative decrease of daily ghee intake and the relative increase in oils.

The Kingdom's daily intake of omega-3 fats was 0.8 g which is 0.21% of the total daily energy intake. The percentages of the daily intake of the governorates ranged from 0.12 to 0.22. The Kingdom's percentage of the daily intake in 2006/2007 was 0.20 (Alkurd, 2011), which is similar to the value obtained in the present study. However the AMDR recommendation of 0.6-1.2% is much higher than the present daily intake for n-3 fats.

Regarding the daily intake of omega-6 fats, it was 8.2 g (2.2% of the total daily energy intake), with percentages of governorates daily intake, ranging from 1.4% in Ma'an to 2.3% in Amman. It is noteworthy that the 2006/2007 value of 2.3% (Alkurd, 2011) is similar to the present value. Again, like in omega-3 daily intake, the omega-6 AMDR recommendation (5-10%) is much higher.

When we look at the ratio of n-6:n-3 in the present survey, we find that it is 10:1, which is within the recommended ratio of these fatty acids, i.e., from 5:1 to 10:1 (Jones and Kubow, 2006). The ratio in the 2006/2007 survey was 12:1 (Alkurd, 2011), which means that the present consumption pattern of n-3 and n-6 fats has improved. High amounts of n-6 fats may exert adverse effects on the function of vascular endothelium or stimulate production of proinflammatory cytokines. Thus, a low ratio of n-6 to n-3 fats is recommended (Basu *et. al*., 2006; Kelly and Sabate, 2006; Gebauer *et. al*., 2006).

Table 4 shows the daily *per capita* estimated intakes of dietary fiber and cholesterol in the whole country and the governorates in Jordan. The *per capita* estimated cholesterol intake ranged from 180 mg (in Ma'an) to 345 mg (in Tafilah), whereas the Kingdom's intake was 303 mg. Only three governorates consumed more than 300 mg of cholesterol daily. The Kingdom's daily cholesterol intake in the 2006/2007 survey was 204 mg/day (Alkurd, 2011), which is lower than both the present daily intake and the upper healthy recommended daily intake of 300 mg/day.

Table 4. Daily *per capita* estimated intakes of dietary fiber and cholesterol in the whole country and governorates in Jordan based on JHEIS 2010 and JHEIS 2006/2007[1]

Governorate	Dietary fiber (g)		Dietary fiber g/ 1000 kcal consumed		Cholesterol (mg)	
	2010	**2006/ 2007**	**2010**	**2006/ 2007**	**2010**	**2006/ 2007**
Amman	24.3	24.0	7.3	8.2	326	241
Balqa	23.9	26.1	8.2	8.5	277	154
Zarqa	23.9	22.6	7.3	7.8	301	218
Madaba	35.3	25.2	8.1	7.6	294	232
Irbid	22.5	26.9	7.2	8.1	297	234
Mafraq	25.9	21.6	7.9	7.5	254	181
Jarash	31.3	28.0	7.3	9.2	343	207
Ajloun	19.1	25.1	6.9	7.7	258	228
Karak	29.3	21.6	7.5	7.0	279	194
Tafilah	20.5	20.1	6.3	7.4	345	200
Ma'an	21.1	25.4	6.9	8.3	180	175
Aqaba	21.8	21.0	7.7	7.6	224	181
Whole country	24.1	24.0	7.2	7.9	303	204
% of change (Kingdom from 2006/2007 to 2010)	+0.4		-9.7		+48.5	

[1]References: DOS 2012; DOS 2008; Alkurd, 2011

As for the dietary daily fiber intake of the Kingdom, it was 7.2 g/1000 kcal. This intake is nearly half of the recommended amount of 14 g/1000 kcal (USDA, 2010), with values in the governorates ranging from 6.3 g (in Tafilah) to 8.2 g (in Balqa). The 2006/2007 dietary fiber daily intake of the Kingdom was 7.9 g/1000kcal, which is higher than the present figure. This indicates that the daily fiber intake is low and needs to be doubled. The best and most practical way of increasing dietary fiber intake can be achieved through consuming more vegetables, fruits, and whole cereals and legumes. The low consumption of fiber-rich foods was also reported in other countries of the region (Musaiger, 2002).

Table 5 presents the estimated intakes of four mineral elements related to cardiovascular diseases, namely sodium, potassium, calcium and magnesium. The sodium's daily intake is 6478 mg for the Kingdom, which is more than 4 fold of the AI of sodium (1500 mg) (IOM.2002/2005). This intake is 15% lower than that of the 2006/2007 (7623 mg) (Alkurd, 2011). The present daily intake of the governorates ranged from 4589 mg in Balqa to 15614 mg in Jerash. This is in agreement with other reports indicating that the sodium content in the Arab Middle East diet is high (Musaiger, 2002).

However, it is noteworthy that around 54% of the sodium figure in the JHEIS survey came from the table salt daily intake of 3.11Kg/capita/year, which is equivalent to 3400 mg/capita/day. It is known that not all of the purchased sodium is ingested since part of it is discarded with brine water in many home processed foods, such as boiled white cheese, pickled olives and pickled vegetables. These home processing and preparation practices are common in Jordan. This might partly explain the odd high figure of the sodium daily intake calculated in this budget survey for Jerash

Table 5. Daily *per capita* estimated intake (mg) of some hypertension-related minerals in the whole country and governorates in Jordan based on JHEIS 2010 and JHEIS 2006/2007[1]

Governorate	Sodium		Potassium		Calcium		Magnesium	
	2010	2006/2007	2010	2006/2007	2010	2006/2007	2010	2006/2007
Amman	5926	7062	3162	3188	686	873	308	307
Balqa	4589	10877	2954	3058	552	909	285	297
Zarqa	7004	6767	3154	3018	629	824	326	295
Madaba	9865	8896	3679	3522	778	917	383	341
Irbid	5397	9155	3000	3672	740	949	306	359
Mafraq	6550	6556	2952	2740	546	753	304	267
Jarash	15614	8537	3868	3522	740	916	396	327
Ajloun	5480	9600	2523	3574	501	919	260	332
Karak	8022	4747	3301	2776	634	688	339	282
Tafilah	7637	6281	2610	2812	461	654	259	257
Ma'an	6632	7232	2502	3049	392	793	241	286
Aqaba	7356	5762	2600	2624	495	747	274	274
Whole country	6478	7623	3030	3130	627	829	305	302
% of change (Kingdom from2006/2007 to2010)	-17.7		-3.3		-32.2		+1.0	

[1]References: DOS 2012; DOS 2008; Alkurd, 2011

governorate, which is famous for dairy processing and vegetable pickling. The amount of the table salt given for Jerash in the present survey is 11.7 Kg/caput/year (in comparison with only 3.11Kg for the Kingdom) (Dos, 2012). In spite of this, we still find that the daily sodium intake by Jordanians is high, and more accurate methods of assessment of the sodium daily intake, such as 24-hr urine collection method, is suggested (Ji *et. al*., 2012).

The Kingdom's average daily intake of potassium is 3030 mg, which is 35.5% lower than the AI (4700 mg) (IOM, 2002/2005) and lower than the 2006/2007 daily intake (3130 mg) (Alkurd, 2011). There is a wide range in the potassium daily intake among the different governorates (from 2502 mg in Ma'an to 3868 mg in Jerash). This low daily potassium intake may be critical as an indicator of hypertension especially when the high daily sodium intake and the low calcium and magnesium daily intakes are taken into consideration (Ji *et. al*., 2012; Raymond and Couch, 2012). Since plant foods (fruits and vegetables) are rich sources of potassium and magnesium, it is important to encourage their consumption in the Jordanian diet in an attempt to decrease the incidence of cardiovascular diseases.

The Kingdom's calcium daily intake is 627 mg, which is 37% lower than the AI (1000 mg) (Table 5), with a wide range in the governorates (from 392 mg in Ma'an to 778 mg in Madaba). Also the Kingdom's daily intake of magnesium (305 mg) is lower than the AI for males (400-420 mg) and for females (310-320 mg) (IOM, 2002/2005). The intake of the governorates ranged from 241 mg in Ma'an to 396 mg in Jerash. It is noticed that even the highest daily intake was lower than the adult male recommendations. The magnesium's daily intake in this survey is similar to that of 2006/2007 intake (Alkurd, 2011).

4. Conclusion

In this study, the Kingdom's estimated daily intakes of total fat, saturated fat, polyunsaturated fat, *trans* fat and cholesterol were within the recommendations. However, the estimated daily intakes of energy and sodium were very high. On the other hand, the estimated daily intakes of monounsaturated, omega-3, omega-6 fats, dietary fiber, potassium, calcium and magnesium were lower than the recommendations. Thus, the present study highlights the unhealthy daily intakes of many dietary factors favorable to CVD, especially hypertension, such as energy, sodium, monounsaturated and omega-3 fats, dietary fiber, potassium, calcium and magnesium.

Acknowledgement

The authors would like to thank the Deanship of Academic Research at The University of Jordan for the financial support, the Jordanian Department of Statistics for providing the authors with the data on Household Expenditure and Income Survey (2010) and Dr. Hiba Al-Sayyed for data entry to the software program.

References

Alkurd R. 2011. Estimated intakes of fats, cholesterol, fiber, sodium, calcium and potassium in Jordan. *Austral J Basic Appl Sci.,* **5**(12) 3171-3178.

Al-Nsour M, Zindah M, Belbeisi A, Hadaddin R, Brown DW and Walke H. 2012. Prevalence of selected chronic, noncommunicable disease risk factors in Jordan: results of the 2007 Jordan Behavioral Risk Factor Surveillance Survey. *Prev Chronic Dis* ***9****:E25*. http://www.cdc.gov/pcd/issues/2012/ 11_0077.htm

Basu A, Devaraj S and Jialal I. 2006. Dietary factors that promote or retard inflammation. *Arterioscler Thromb Vasc Biol*, **26**:995-1001.

Bo S and Pisu E. 2008. Role of dietary magnesium in cardiovascular disease prevention, insulin sensitivity and diabetes. *Curr Opin Lipidol*, **19**(1):50–56.

Brown DW, Mokdad AH, Walke H, As'ad M, Al-Nsour M, Zindah M, Arqoob K, Belbeisi A. 2009. Projected burden of chronic, noncommunicable diseases in Jordan [letter]. *Prev Chronic Dis* **6**(2):A78.

Cabo J, Alonso R and Mata P. 2012. Omega-3 fatty acids and blood pressure. *Br J Nutr***, 107 Suppl. S2,** S195-S200**.**

Cunha AB, Umbelino B, Correia ML and Neves MN. 2012. Magnesium and Vascular Changes in Hypertension. *Int. J Hypertens*. 2012: 754250. Published online 2012 February 29. doi: 10.1155/2012/754250

DOS (Department of Statistics), 2012. Household Expenditures and Income Survey 2010. DOS, Amman-Jordan.

DOS (Department of Statistics), 2008. Household Expenditures and Income Survey 2006. DOS, Amman-Jordan.

Elmadfa I and Kornsteiner M. 2009. Fats and fatty acid requirements for adults. *Ann Nutr Metab*. **55**:56–75.

Erkkilä AT and Lichtenstein AH. 2006. Fiber and cardiovascular disease risk: how strong is the evidence? *J Cardiovasc Nurs*, **21**:3–8.

Fahed AC, El-Hage-Sleiman AM, Farhat TI and Nemer GM. 2012. Diet, genetics, and disease: a focus on the Middle East and North Africa region. J Nutr Metabol 2012, Article ID 109037, 19 pages. doi:10.1155/2012/109037

Food Processor SQL, 2008**. Food Processor Nutrition and Fitness Software.** Food Processor SQL Inc., Salem, OR, USA.

Gallagher ML. 2012. Intake: the nutrients and their metabolism. In: Mahan L, Escott-Stump S. and Raymond J. (Eds.), **Krause's Food and Nutrition Care Process**. **13th** ed. (pp. 42-44). Elsevier. USA.

Gebauer SK, Psota TL, Harris WS and Kris-Etherton PM. 2006. n-3 fatty acid dietary recommendations and food sources to achieve essentiality and cardiovascular benefits. *Am J Clin Nutr*, **83**(**6 Suppl**):1526S-1535S.

Hatzistavri LS, Sarafidis PA, Georgianos PI, Tziolas IM, Aroditis CP, Zebekakis PE, Pikilidou MI and Lasaridis AN. 2009. Oral magnesium supplementation reduces ambulatory blood pressure in patients with mild hypertension. *Am J Hypertens*, **22**(10):1070–1075.

He K, Liu K, Daviglus ML, Morris SJ, Loria CM, Van Horn L, Jacobs DR Jr and Savage PJ. 2006. Magnesium intake and incidence of metabolic syndrome among young adults. *Circulation*, **113**(13):1675–1682.

Hu FB. 2008. Globalization of food patterns and cardiovascular disease risk. *Circulation*. 118:1913–4. doi: 10.1161/ CIRCULATIONAHA.108.808493

IOM (Institute of Medicine). Dietary Reference Intakes for Calcium and Vitamin D. 2010. Web site http://www.iom.edu/Reports/2010/Dietary-Reference-Intakes-for-Calcium-and-Vitamin-D.aspx. Accessed October 31, 2013.

IOM (Institute of Medicine), 2002/2005. Panel on Macronutrients and Standing Committee on the Scientific Evaluation of Dietary Reference Intakes. Macronutrients and healthful diets. Dietary reference intakes for energy, carbohydrates, fiber, fats, fatty acids, cholesterol, proteins, and amino acids. Washington, DC: National Academies Press, 769-879.

IOM (Institute of Medicine), 2001. Dietary Reference Intakes for Energy, Carbohydrate, Fiber, Fat, Fatty Acids, Cholesterol, Protein, and Amino Acids. From:www.nal.usda.gov/fnic/DRI/DRI_Energy/energy_full_report.pdf

Ji C, Sykes L, Paul C. Dary O; Legetic B; Campbell NRC and Cappuccio FB. 2012. Systematic review of studies comparing 24-hour and spot urine collections for estimating population salt intake. *Rev Panam Salud Publica*. ,**32(4)**: 307–15.

Jones P and Kubow S. 2006. Lipids, sterols, and their metabolites. In: Shils ME, Shike M, Ross AC, Caballero B and Cousins RJ (Eds.), **Modern Nutrition in Health and Disease**, 10th ed. Lippincott Williams & Wilkins.

Karppanen H, Karppanen P and Mervaala E. 2005. Why and how to implement sodium, potassium, calcium, and magnesium changes in food items and diets? *J Hum Hypertens*, **19**, S10–S19.

Kelly JH and Sabate J. 2006. Nuts and coronary heart disease: an epidemiological prospective. *Br J Nutr*, **96**: S61.

Kris-Etherton PM, Taylor DS, Yu-Poth S, Huth P, Moriarty K, Fishell V, Hargrove RL, Zhao G and Etherton TD. 2000. Polyunsaturated fatty acids in the food chain in the United States. *Am J Clin Nutr*, **71**(**1 Suppl**):179S-188S.

Lamotte M, Annemans L, Kawalec P and Zoellners Y. 2006. A multi-country health-economic evaluation of highly concentrated n-3 polyunsaturated fatty acids in the secondary prevention after myocardial infarction. *Herz*, **31**(**Suppl 3**):74–82.

Lichtenstein AH, Appel LJ, Brands M, Carnethon M, Daniels S, Franch HA, Franklin B, Kris-Etherton P, Harris WS, Howard B, Karanja N, Lefevre M, Rudel L, Sacks F, Van Horn L, Winston M and Wylie-Rosett J. 2006. Diet and lifestyle recommendations revision 2006: a scientific statement from the American Heart Association Nutrition Committee. *Circulation*, **114**:82–96.

Ma S, Park B, Yang J, Jung E, Yeo Y, Whang Y, Chang S, Shin H, Kang D, Yoo K and Park S. 2012. Interaction of Body Mass Index and Diabetes as Modifiers of Cardiovascular Mortality in a Cohort Study**.** *J Prev Med Public Health*, **45**(6): 394-401. doi: 10.3961/jpmph.2012.45.6.394

Madanat HN, Troutman KP and Al-Madi B. 2008. The nutrition transition in Jordan: the political, economic and food consumption contexts. *Promot Educ*, **15**(1):6-10. doi: 10.1177/1025382307088092.

Micha R and Mozaffarin D. 2009. Trans fatty acids: effects on metabolic syndrome, heart disease and diabetes. *Nat Rev Endocrino*, **5**:335.

Mozaffarian D and Stampfer MJ. 2010. Removing industrial *trans* fat from foods. Rendering food injurious to health. *BMJ*. 340:c1826. doi: 10.1136/bmj.c1826.

Musaiger AO and Al-Hazzaa HM. 2012. Prevalence and risk factors associated with nutrition-related noncommunicable diseases in the Eastern Mediterranean region. *Intern J Gen Med* **5**: 199–217.

Musaiger AO, Takruri HR, Hassan AS and Abu-Tarboush H. 2012. Food-based dietary guidelines for the Arab Gulf countries. *J Nutr Metabol* **2012**. doi:10.1155/2012/905303.

Musaiger AO. 2006. **Food Composition Tables of Common Foods of Gulf Cooperation Council Countries** *(in Arabic)*, Arab Centre for Nutrition, Manama, Bahrain.

Musaiger AO. 2002. Diet and Prevention of Coronary Heart Disease in the Arab Middle East countries. *Med Princ Pract* **11(Suppl 2**):9–16. (DOI:10.1159/000066415)

National Heart, Lung and Blood Institute. 2001. The Practical Guide Identification, Evaluation, and Treatment of Overweight

and Obesity in Adults. From: www.nhlbi.nih.gov/guidelines/obesity/prctgd_c.pdf.

Pellet PL and Shadarevian S. 1970. **Food Composition Tables for Use in the Middle East.** AUB, Beirut, Lebanon.

Popkin BM. 2002. The shift in stages of the nutrition transition in the developing world differs from past experiences! *Publ Health Nutr*. **5**(1A):205–14.

Raymond JL and Couch SC. 2012. Medical nutrition therapy for cardiovascular disease. In: Mahan L, Escott-Stump S and Raymond J (Eds.), **Krause's Food and Nutrition Care Process**. **13th** ed. Elsevier. USA.pp 742-781.

Rolfes SR, Pinna K and Whitney E. 2012. **Normal and Clinical Nutrition.** 9th ed. Wadsworth, Cengage Learning.

Scarborough P, Nnoaham K, Clarke D and Rayner M. 2011. Differences in coronary heart disease, stroke and cancer mortality rates between England, Wales, Scotland and Northern Ireland: the role of diet and nutrition. *BMJ Open*. 1:e000263. doi: 10.1136/bmjopen-2011-000263.

Simopoulos AP. 2008. The importance of the omega-6/omega-3 fatty acid ratio in cardiovascular disease and other chronic diseases. *Exp Biol Med* (Maywood), **233**:674–688.

Stovall R, Hwang M, Christiansen M and Sa'ad Al-Deen, JJ. 2013. Nutritional Status of 9th Grade Students in Amman, Jordan. *J Glob Health Perspec*. From: http://jglobalhealth.org/wp-content/uploads/2013/02/JGHP_Stovall_Rachael.pdf

Takruri H, Alkurd R and Faris M. 2011. Trends of energy and macronutrient intake of Jordanians (*in Arabic*). *J Saudi Soc Food and Nutr*, **6** (2): 40-54.

Touyz R, Milne FJ and Reinach SG. 1992. Intracellular Mg^{2+}, Ca^{2+}, Na^{2+} and K^{+} in platelets and erythrocytes of essential hypertension patients: relation to blood pressure. *Clin Exper Hypertens*, **14**(6):1189–1209**)**.

USDA. 2010. United States Department of Agriculture Dietary Guidelines for Americans. http://www.cnpp.usda.gov/Publications/DietaryGuidelines/2010/PolicyDoc/ExecSumm.pdf

US Census Bureau. 2004[a]. Population Estimates.

US Census Bureau. 2004[b]. International Data Base.

USDA. 2002/2005. Dietary Reference Intakes for Energy, Carbohydrate, Fiber, Fat, Fatty Acids, Cholesterol, Protein, and Amino Acids.

Uza G, Pavel O, Uza D, and Valaicu R. 1987. Hypomagnesemia in patients with essential arterial hypertension. *Magnesium-Bull*, **9**:177–180.

Wertz PW. 2009. Essential fatty acids and dietary stress. *Toxicol Ind Health*, **25**:279.

WHO. 2012. New data highlight increases in hypertension, diabetes incidence.

WHO. 2007. Prevention of Cardiovascular Disease. Pocket Guidelines for Assessment and Management of Cardiovascular Risk.

JJBS

Volume 7, Number 2, June .2014
ISSN 1995-6673
Pages 127 - 132

Jordan Journal of Biological Sciences

The Ability of Vitamin A, Alone or in Combination with Vitamins C and E, in Ameliorating the Side Effects of Penicillin and Streptomycin on Hepatic Damage in Guinea Pigs

Mohammed A.Y. Al-Eryani[1] , Fatma M. H. Shediwah[3], Mohammed S.A. Al-Awar[1,*] , Elias M.A. Salih[2] and Elham A. S. AL- Shaibani[3]

[1] *Department of Biology, Faculty of Education, University of Amran,*

[2] *Department of Biology, Faculty of Education, University of Aden,*

[3] *Department of Biology, Faculty of Science, University of Sana'a, Yemen*

Received: January 5, 2014 Revised: February 12, 2014 Accepted: February 19, 2014

Abstract

The administration of vitamin A (10000 IU/kg b.w), vitamin A, C and E (10000 IU/kg, 100 mg/kg 100 mg/kg b.w), penicillin (50000 IU/kg b.w), penicillin + vitamin A, penicillin + vitamin A, C and E, streptomycin (50 mg/kg b.w), streptomycin + vitamin A, streptomycin + vitamins A, C and E for 30 days caused a significant increase in the levels of AST, ALT and ALP, and also caused a significant decrease in the levels of total protein and albumin in penicillin and streptomycin treated groups. However, co-administration of penicillin and streptomycin with vitamin A alone or in combination with vitamins C and E ameliorated the harmful effects of penicillin and streptomycin in most of the tested parameters. Vitamin A, either alone or in combination with vitamin C and E, has a protective effect on the histological changes of liver tissues induced either by penicillin or streptomycin administration. The result of the present work reveals for the first time that vitamin A alone or in combination with vitamin C and E plays an important role as cytoprotective compounds on hepatic damage in guinea pigs. Hence, further studies on the possible uses of vitamins as protective compounds during treatment with antibiotics are needed.

Keywords: Vitamin A Alone, Vitamins A, C And E, Penicillin, Streptomycin, Histological And Biochemical Study.

1. Introduction

Antibiotics constitute a family of drug, which taken as a group, represents one of the most frequently prescribed around the world. Thus, not surprisingly antibiotic list on the top of causes of drug induced many side effects (Maliha *et al.*, 2009). The effects of penicillin and streptomycin on histological structure and function of liver are well studied (Al-Awar *et al*., 2013 and Akande *et al*., 2012). It has been reported that effects of aminoglycoside and β-Lactams are mainly due to the generation of an excessive amount of reactive oxygen species (ROS), resulting in the detrimental effects of the cellular antioxidant defense system as well as the enhancement of the lipid peroxidation (LPO) process (Westphal *et al*., 1994; Sha and Schacht 1999; and Goldstein and Ishak 1999).

Antioxidants protect key cell components from damage by neutralizing the free radicals (Dekkers *et al*., 1996). Antioxidants that occur naturally in the body or that are consumed through the diet may block damage to cells (Cherubini *et al.*, 2005). Therefore, supplementation of antioxidants can be considered as the alternative method for chelation therapy. In fact, several studies demonstrated that the cellular antioxidant activity is reinforced by the presence of dietary antioxidants (Prior and Cao, 2000). Accordingly, interest has recently grown in the role of natural antioxidants used as a strategy to prevent oxidative damage as a factor in the pathophysiology of various health disorders (Shireen *et al.*, 2008). Among antioxidants, vitamins A, C and E have the ability to counteract free radicals and protect the structure and function of proteins, DNA and chromosomes against oxidation injury, and they are the most powerful in reducing storage and toxicity of reactive oxygen species (Seham and Awatef, 2008). In this regard, studies on vitamins A, C and E added to diet are promising, mainly due to their antiradical activity, indicating that they could provide an important dietary source of antioxidants. Many studies indicate to protective effects of vitamins A, C and E against many alteration caused by organophosphate insecticides and some medicines that induced hepatotoxicity (Velanganni and Balasundaram, 2003; and Ukpanukpong *et al*., 2013). No data were available in the literature related to the

* Corresponding author. e-mail: momed.sadeg@gmail.com.

protective effect of vitamin A alone or in combination against the side effect of antibiotics (penicillin and streptomycin) on hepatotoxicity. The present study is the first study undertaken to investigate the ability of vitamin A alone or in combination with vitamins C and E to ameliorate the side effect of antibiotics (penicillin and streptomycin) on hepatic damage in guinea pigs.

2. Materials and Methods

2.1. Animals

For this experiment, eighty male guinea pigs (5-6 months old) weighing between 800 - 900 g were obtained from the Zoo, Sana'a, Yemen. The animals were housed in plastic cages in the animal house of the Department of Biology- Faculty of Science- Sana'a University, under standard conditions in room temperature, fed a standard laboratory diet and water *ad libitum*. The animals were allowed to acclimatize to the laboratory environment for 30 days. All animal experiments were carried out in accordance with the Guide for the Care and Use of Laboratory Animals published by the National Institute of Health (NIH, 1978), and were approved by the Animal Experiments Local Ethics Committee at the Zoo, Sana'a, Yemen.

2.2. Drugs and Chemicals

Penicillin (Procaine G penicillin) and streptomycin were obtained from Ave Group-USA-Colombia-Mexico. Vitamin A (Retinol Assay:99 Appearance: Slightly yellow solid Formula: C20H30O Molecular Weight: 286.50) was supplied by Look for chemical (Hangzhou, China). Vitamin C ((L-) ascorbic acid Assay: 99%-100% Appearance: White crystalline powder Formula: C6H8O6 Molecular Weight: 176.14) was supplied by Carlo Erbo (Milano, Italy). Vitamin E (DL-alpha- tocopherol acetate Assay: 96% Appearance: low yellow powder Formula:C29H50O2 Molecular Weight: 430.71) was supplied by Merck (Germany).

2.3. Experimental Animals

Eighty adult male guinea pigs were divided randomly into 9 groups. Animals that received vitamins were administrated orally, whereas those that received antibiotics were administered intraperitoneally (i.p). Penicillin, streptomycin and vitamin C were dissolved in distilled water, while vitamin A and vitamin E were dissolved in corn oil. Treatments were carried out over a period of 30 days. Treatment groups were as follows:

Group 1: 10 animals received 0.5 ml/kg b.w corn oil orally and served as control.
Group 2: 5 animals treated with vitamin A (10000 IU/kg b.w).
Group 3: 5 animals treated with vitamins A, C and E (10000 UI/kg ,100 mg/kg & 100 mg/kg b.w), respectively.
Group 4: 10 animals treated with penicillin (50000 IU/kg b.w).
Group 5: 10 animals treated with penicillin (50000 IU/kg b.w) + vitamin A (10000 UI/kg b.w).
Group 6: 10 animals treated with penicillin (50000 IU/kg b.w) + vitamins A, C and E (10000 UI/kg ,100 mg/kg & 100 mg/kg b.w), respectively.
Group 7: 10 animals treated with streptomycin (50 mg/kg b.w).
Group 8: animals treated with streptomycin (50 mg/kg b.w) + vitamin A (10000 UI/kg b.w).
Group 9: 10 animals treated with streptomycin (50 mg/kg b.w) + vitamins A, C and E (10000 UI/kg ,100 mg/kg & 100 mg/kg b.w), respectively.

2.4. Collection the Blood and Tissue

24 h after last administration, animals of each group were autopsied; blood samples were taken from the heart and collected into sterile tubes centrifuged at rpm for 20 min, and serum was separated for biochemical tests. The liver of each guinea pig removed, small pieces of liver were taken, then fixed in 10% neutral formalin for 24 hours and were kept in alcohol for the tissue preparation.

2.5. Estimation of Liver Function

Aspartate Amino Transferase (AST), Alanine Amino Transferase (ALT), Alkaline Phosphatase (ALP), Total protein and albumin were measured by spectrophotometry in serum using Spinreact commercial kits.

2.6. Histological Studies Liver

The liver specimens of each guinea pig were dehydrated in series of alcohol concentrations 80%, 90% and 100%, then cleared in xylene, embedded in paraffin wax at 58 °C. Blocks were cut at 4-5 µm thickness by using rotary microtome (Leica, Germany) and stained with hematoxylin and eosin (Humason 1979) for histopathological examination under light microscope.

2.7. Statistical Analysis

The data were analyzed using SPSS 16.0 for windows. A statistical analysis was performed using one-way Analysis of Variance (ANOVA), followed by Fisher's Protected Least Significant Difference (PLSD) test as a post hoc test for the comparison between the groups. All values were expressed as means ± SD. Differences were considered significant if $p< 0.05$.

3. Results

3.1. Biochemical results

Data in tables 1 and 2 shows that the treatment of penicillin and streptomycin resulted in a statistically high significant increase in the levels of ALT, AST and ALP in the serum of both treated groups, as compared to the control; this increase was higher in the streptomycin treated guinea pigs.

As shown in tables 1 and 2, the level of the total protein and albumin in the serum of guinea pigs, treated with penicillin and streptomycin, statistically shows a highly significant decrease compared to the control.

Data in tables 1 and 2 also shows that the treatment with vitamin A alone for 30 days in group 2 showed comparable results to the control regarding the levels of AST, ALT, ALP, albumin and total protein. The co-administration of vitamin A with penicillin in group 5 or

with streptomycin in group 8 caused a low significant increase in the level of AST, ALT and ALP, as compared to that of the control group. Meanwhile, results showed a low significant decrease in the level of the total protein and albumin as noticed in the serum of the treated guinea pigs in comparison to that of the control group.

Results in table 2 revealed that the treatment with Vitamins A, C and E in combination (group 3) gave comparable results to those of the control regarding the levels of AST, ALT, ALP, albumin and total protein.

The improvement in the tested biochemical parameters insured that vitamins A, C and E in combination have a protective role against the side effects of penicillin and streptomycin on liver. The co-administration of vitamins A, C and E in combination beside penicillin in group 6 or streptomycin in group 9 resulted in a non-significant increase in the level of AST, ALT and ALP as compared to that of the control group. Meanwhile, a non-significant decrease occurred in that total protein and albumin, as compared to that of the control group, as shown in table 2.

Table 1. The protective effect of vitamin A alone to reduce the adverse effects induced by penicillin and streptomycin on liver function tests of guinea pigs.

Treatment / **Parameter**	**Control C.Oil for 30days**	**Vit A for 30 days**	**Penicillin for 30days**	**Vit A & P for 30 days**	**Streptomycin for 30days**	**Vit A & S for 30days**
AST	22.86±2.49	21.90±0.9	46.88±4.9†††	29.14±2.2$^{\#\#\#\dagger}$	60.35±6.8†††	33.18±3.9***†
IU/L	……..	0.2%	113.6%	32.8%	174.9%	51.2%
ALT	27.82±1.21	29.07±1.8	63.06±5.6†††	34.41±3.5$^{\#\#\#\dagger}$	79.36±3.5†††	38.41±3.4***†
IU/L	……..	6.1%	130.1%	25.5%	189.5%	40.1%
ALP	53.03±2.28	53.69±1.6	78.06±4.3†††	58.44±3.5$^{\#\#\#\dagger}$	88.46±3.4†††	60.11±3.7***†
IU/L	……..	1.4%	47.5%	10.5%	67.2%	13.6%
Total protein	7.64±0.20	7.57±0.13	6.03±0.45†††	7.03±0.36$^{\#\#\#\dagger}$	5.44±0.35†††	7.01±0.26***
g/dl	……..	0.7%	19.8%	6.5%	27.27%	6.8%
Albumin	3.83±0.16	3.60±0.34	29.6±0.18†††	3.42±0.24$^{\#\#\#\dagger}$	2.29±0.12†††	3.08±0.15***
g/dl	……..	4.7%	21.7%	9.5%	39.4%	18.5%

Values are expressed as means ± SD; percentage of difference with control group. Comparisons are made between each group and (†): control group; (#): only penicillin treated group; (*): only streptomycin treated group**.**

Table 2. The protective effect of vitamin A,C and E in combination to reduce the side effects induced by penicillin and streptomycin on liver function tests of guinea pigs.

Treatment / **Parameter**	**Control. C.Oil for 30days**	**Vit A,C, E for 30days**	**Penicillin for 30days**	**Vit A,C, E & P for 30days**	**Streptomycin for 30days**	**Vit A,C, E & S for 30days**
AST	22.86±2.49	20.35±1.2	46.88±4.9†††	22.29±2.7$^{\#\#\#}$	60.35±6.8†††	23.13±2.1***
IU/L	……..	7.3%	113.6%	1.5%	174.9%	5.3%
ALT	27.82±1.21	26.75±1.7	63.06±5.6†††	30.23±2.2$^{\#\#\#\dagger}$	79.36±3.5†††	32.57±3.4***†
IU/L	……..	2.4%	130.1%	10.3%	189.5%	18.8%
ALP	53.03±2.28	53.78±2.0	78.06±4.3†††	52.63±3.0$^{\#\#\#}$	88.46±3.4†††	53.80±3.0***
IU/L	……..	1.6%	47.5%	0.5%	67.2%	1.7%
Total protein	7.64±0.20	7.70±0.20	6.03±0.45†††	7.50±0.14$^{\#\#\#}$	5.44±0.35†††	7.48±0.32***
g/dl	……..	2.4%	19.8%	0.3%	27.27%	0.5%
Albumin	3.83±0.16	3.93±0.41	29.6±0.18†††	3.71±0.20$^{\#\#\#}$	2.29±0.12†††	3.28±0.11***
g/dl	……..	4%	21.7%	1.9%	39.4%	13.2%

Values are expressed as means ± SD; percentage of difference with control group. Comparisons are made between each group and (†): control group; (#): only penicillin treated group; (*): only streptomycin treated group**.**

3.2. Histological Results

Examination of the section in the liver of control guinea pigs (Figure 1a), showed that the hepatic exhibits a normal architecture of the hepatocytes presenting a homogenous cytoplasm and a large spherical nucleus containing one or more nucleolus and a variable amount of dispersed and peripheral heterochromatin. Hepatocytes were arranged in trabecules running radiantly from the central vein and were separated by sinusoid containing kupffer cells. The lumen of sinusoid contained mainly erythrocytes and white blood cells.

Light microscopic examination of the liver after administration of vitamin A alone (Figure 1b) and vitamins A,C and E in combination for 30 days (Figure 2c) revealed a normal picture as in control group.

Histological examination of the liver after administration of penicillin and streptomycin for 30 days showed obvious histological changes in the form of distortion in the hepatic organization, dilatation and congestion of the blood sinusoids and central vein, neutrophils infiltration, hemorrhage, congestion and hyperplasia of the bile duct wall, as well as necrosis, vasodilatation and thickening in the central vein (Figures 2 a, b, c, d and f).

The administration of vitamin A or vitamins A, C and E in combination plus penicillin for 30 days (Figures 3a and 4a), respectively, and the administration of vitamin A or vitamins A, C and E in combination besides streptomycin (Figures 3b and 4b), respectively, revealed a normal structure as in control group.

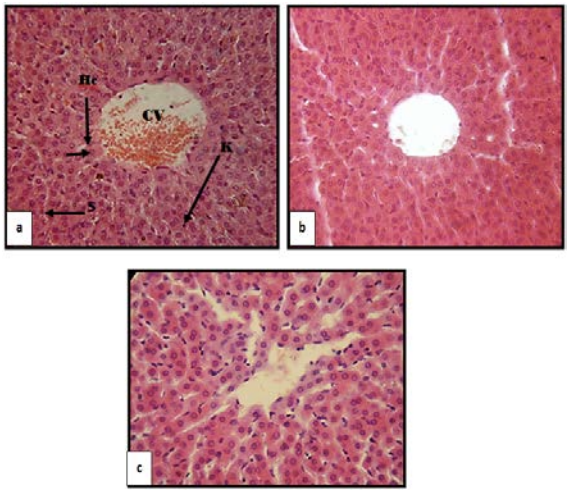

Figure 1. Light micrograph of section in the liver of guinea pigs. **(a):** control group showing a normal architecture without pathological alterations. Hepatocyte (Hc), Spherical nucleus (arrow), Sinusoids (S), Blood vessel (BV), and Kupffer cells (K). **(b):** Vitamin A; **(c):** vitamin A, C and E in combination, showed normal liver structure as in control group. (HE) stain (X400).

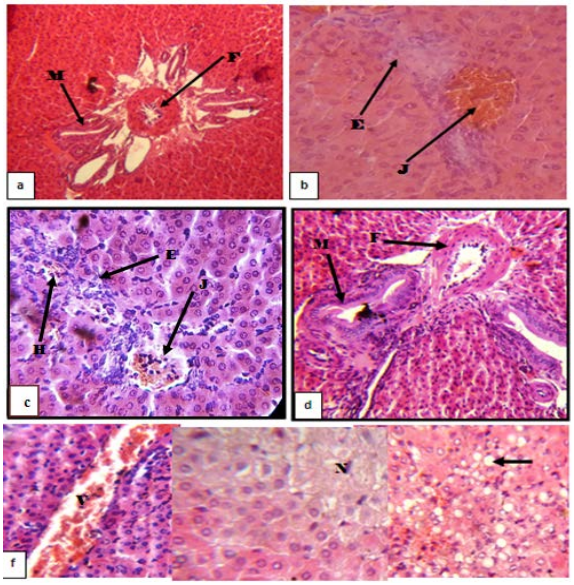

Figure 2. Light micrograph of section in the liver of guinea pigs. **(a,b,f):** Penicillin; **(c,d,f):** Streptomycin; showing obvious histopathological changes. Hemorrhage (H), Congestion (J), Inflammatory cells infiltration (E), Thickening in the central vein (F), Hyperplasia (M), Fatty changes (arrow), Necrosis (N), Vasodilatation (V). (HE) stain (X400).

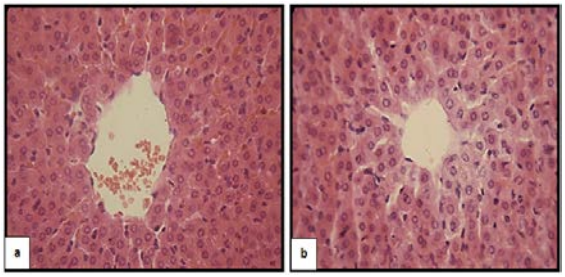

Figure 3. Light micrograph of section in the liver of guinea pigs. **(a):** Penicillin+ vitamin A; **(b)** Streptomycin +vitamin A; showing a normal liver structure as in control group. (HE) stain (X400).

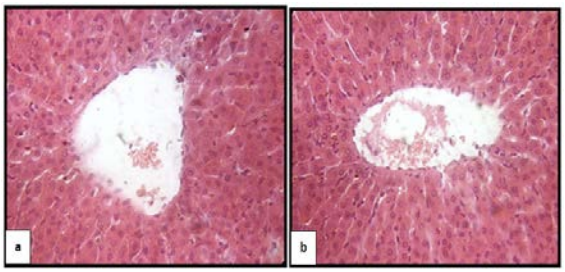

Figure 4. Light micrograph of section in the liver of guinea pigs. **(a):** Penicillin+ vitamins A,C,E in combination; **(b)** Streptomycin + vitamins A,C,E in combination; showing a normal liver structure as in control group. (HE) stain (X400).

4. Discussion

Our results clearly show the hepatotoxic effects of penicillin and streptomycin. The administration of penicillin and streptomycin caused marked increases in the enzymes AST, ALT and ALP as compared to those of the control. These results may indicate degenerative changes and hypo-function of liver (Adebajo *et al.*, 2009) as well as hepatic cell necrosis (Singh *et al.*, 2005), which increases the releasing of these enzymes in the blood stream (Jaramillo-Jurez *et al.*, 2008). Elevated levels of these enzymes in the serum are presumptive markers of drug-induced necrotic lesions in the hepatocytes (Singh *et al.*, 2005). The enhanced susceptibility of hepatocyte cell membrane to drug-induced peroxidative damage might have resulted in an increase releasing of these diagnostic marker enzymes into the systemic circulation. Our observations are highly supported by other studies suggesting the effect of penicillin and streptomycin on liver function tests (Alqadhi 2010; Akande *et al.*, 2012; Al-Awar *et al.*, 2013 and Al-Shaibani *et al.*, 2013).

Our present study also shows that administering penicillin or streptomycin to guinea pigs results in a significant decrease in the level of total protein and albumin compared with the control group. The decrease in the total protein and albumin recorded in the present study is supported by the results reported by Austin *et al.* (1993), Akande *et al.* (2012), Al-Awar *et al.* (2013), and Al-Shaibani *et al.* (2013). The reduction of the total protein and albumin levels indicates that the administration of drugs has caused an impairment of liver function, e.g. its capacity to synthesize albumin from the hepatic parenchyma. Khan *et al.* (2002) reported that there was a differential binding of penicillin and streptomycin with serum albumin, while Salih *et al.* (2008) observed that albumin secretion of gel entrapped hepatocytes was reduced by penicillin and streptomycin.

The mechanism of penicillins and aminoglycosides induced hepatotoxicity is found to be mediated through oxidative stress by free radical that cause damage to hepatocytes (Goldstein and Ishak (1974) and Sherlock and Dooley (2002)). AST, ALT and ALP increase in hepatic damage due to leakage of enzymes from the damaged hepatocytes into vascular compartment. Liver damage leads to a decrease in synthetic capability, leading to a fall in serum total protein and albumin levels (Sherlock and Dooley, 2002).

The present investigation clearly demonstrated that the injection of penicillin and streptomycin to guinea pigs had induced conspicuous alteration in the histological structure on the liver tissue in the treated guinea pigs. Our results are in agreement with those of Austin *et al.*, (1993), Al-Awar *et al.* (2013) and Al-Shaibani *et al.* (2013).

Histopathological changes in liver cells following injection of penicillin were the marked changes occurring in the liver in this study. This feature could be explained according to the suggestions of both Tayala *et al.* (2007) and Al-Awar *et al.* (2013). Al-Saibani *et al.* (2013) reported that histopathological changes in liver cells due to free radical generating and free radical scavenging enzymes may be disturbed and leading to disrupt signal transduction pathway and increase the cellular permeability by acting on the membrane phospholipids, resulting into a significant hepatic tissue injury.

The antioxidant activities are related to a number of different mechanisms, such as free radical-scavenging, hydrogen- donation, singlet oxygen quenching, metal ion chelation, and acting as a substrate for radicals such as superoxide and hydroxyle (Robards *et al.*, 1999). In the present investigation it was observed that the activities of these enzymes were reduced after the treatment by vitamin A alone or vitamin A in combination with vitamins C and E, when compared to penicillin and streptomycin treated guinea pigs alone. The penicillin and streptomycin induced oxidative stress has lowered, on hypothesis to explain the beneficial effects of vitamins A, C and E in ameliorating biochemical parameters and histological changes is that vitamins A, C and E considered antioxidant, scavenging and eliminating free radicals (Awodele *et al.*, 2010; Al-Awthan *et al.*, 2012). Recently, it has been found that vitamins A, C and E lead to increasing the levels of total protein and albumin in biological fluids and to reduce liver enzymes, such as AST, ALT and ALP in serum (Al-Awthan *et al.*, 2012; Ganesh *et al.*, 2012). vitamins A, C and E showed a significant improvement in liver tissues. Our results showed that vitamin A alone or in Combination with vitamins C and E administration decreased these histopathological changes. The structure of liver and hepatocytes appearance were more or less similar to control group as well its function. Vitamin A, in Combination with vitamins C and E, was more effective than vitamin A alone, which is consistent with Tarladacalisir *et al.* (2005), Seham and Awatef (2008), Awodele *et al.* (2010) and Ukpanukpong *et al.* (2013) . In conclusion, we suggest that a combination of vitamin A with vitamins C and E is more effective than vitamin A alone for ameliorating the side effects of antibiotics (penicillin and streptomycin) on hepatic damage. Also, the present study concludes that the pathological changes in biochemical parameters as well as liver tissue structure are higher in the streptomycin treated guinea pigs than in the penicillin treated groups.

References

Adebajo AC, Walewa EO, Obuotor E M,Ibikunle GF, Omisore NO and Adewunmi CO. 2009. Pharmacological properties of the extract and some isolated compounds of *Clausenalansium* stem bark: anti-trichomonal, antidiabetic, anti-inflammatory, hepatoprotective and antioxidant effects. *J Ethnopharmacol*. **122(1):**10–19.

Akande T, Balogun ST and Gabriel O. 2012. The effects of penicillin streptomycin on liver aminotransferases, alkaline phosphatase and total serum protein in rabbits (*Orcytolagusconiculus*). *J Appl Pharm Sci*., **2**: 32-35.

Al-Awar MS, AL- Shaibani EA, Salih EM, Al-Eryanim MA. 2013. The protective effect of nabk honey against pathological effects of penicillin and streptomycin on histological structure and functions of guinea pigs liver. *J Appl Pharm Sci.*, **3 (4 Suppl 1)**: S1-S6.

Al-Awthan YS, Al-Douis MA, El-Sokkary GH and Aglan EA. 2012. Dimethoate-induced oxidative stress and morphological changes in the liver of guinea pig and the protective effect of vitamin C and E. *Asian. J. Biol.Sci*., **5 (1):** 9-19.

Alqadhi Y.A. 2010. The effect of the usualand extreme use of Antibiotics on the immune –indicators and liver and Kidney Functions in experimental animals. Thesis M.Sc. Department of Biology. Faculty of Education Aden. University of Aden.

AL-Shaibani EA, Alarami AM, Al-Awar MS, Salih EM and Al-Eryani MA. 2013. Antioxidant protective effect of vitamin E in penicillin and streptomycin-induced hepatotoxicity in guinea pigs. *J Agricol Biol. Sci.,* **8 (7):** 546-554.

Austin H, Budowsky J, lane J and Chilton W. 1993. Reactions following to the use of penicillin. A controlled study. *J Aller*., **24 (2):** 164 – 171.

Awodele OA, Akintonwa V, Osunkalu O and Coker HB. 2010. Modulatory activity of antioxidants against the toxicity of rifampicin *in vivo*. *Rev Inst Med Trop Sao Paulo,* **52**: 43-46.

Cherubini A,Vigna GB, Zuliani G, Ruggiero C, Senin U and Fellin R. 2005. Role of antioxidants in atherosclerosis: epidemiological and clinical update. *Curr Pharm Dis*., **11:**2017-2032.

Dekkers JC, Van Doornen LJ and Kemper CG. 1996. The role of antioxidant vitamins and enzymes in the prevention of exercise-induced muscle damage. *Sports Med.*, **12**: 213-238.

Ganesh E, Chowdhury A, Malarvani T and Ashok vardhan N. 2012. Hepatoprotective effect of Vitamin – E & C in Albino rats. *Int J Advanced Life Sci*., **3**: 87-95.

Goldstein LI and Ishak KG. 1999. Hepatic injury associated with penicillin therapy. *Arch Pathol*., **98**: 114–117.

Humason GL. 1979. **Animal Tissue Techniques**. 2nd ed. Freeman W.H, and Company. p. 661.

Jaramillo-Jurez F, Rodrguez-Vzquez ML, Rincn-Snchez AR, Consolacin-Martnez M, Ortiz GG and Liamas J. 2008. Acute renal failure induced by carbon tetrachloride in rats with hepatic cirrhosis. *Ann. Hepato*., **7(4):**331–338.

Khan MA, Muzammil S and Musarrat J. 2002. Differential binding of tetracyclines with serum albumin and induced structural alterations in drug-bound protein. *Int J Biol Macromol.,* **30 (5):** 243–249.

Maliha S S, Shahed MJ, Janker MN, Abasi KW and Nigm-Rahman AE. 2009. Clinical and experimental evidences in antibiotics side effects and toxicity associate with overdose and long-term of use. *Pharmacol Assoc J*., **6(3)** 23-31.

Prior RL and Cao G. 2000. Antioxidant phytochemicals in fruits and vegetables; diet and health implications. *Hortic Sci*., **35:** 588–592.

Robards K, Prenzler PD, Tucker G,Swatsitang P and Glover W. 1999. Phenolic compounds and their role in oxidative processes in fruits. *Food Chem*. **66**:401–436.

Salih SB, Kharal M, Qahtani M, Dahneem L and Nohair S. 2008. Acute interstitial nephritis induced by intermittent use of rifampicin in patient with brucellosis. *Saudi J. Kidney Dis. Transpl.,* **19 (3):** 450–452.

Seham HR and Awatef AM. 2008. Vitamin A against the acetaminophen- induced toxicity in the renal cortex of albino rats. *Egypt J Histol*., **31 (2):**321- 331.

Sha SH and Schacht J. 1999. Stimulation of free radical formation by aminoglycoside antibiotics. *Hear. Res*. **128**: 112-118.

Sherlock S and Dooley J. 2002. Drugs and Liver. In: **Diseases of the Liver and Biliary System**, 11th ed. Blackwell Science: Oxford. UK. Malden. MA. Pp.335-363.

Shireen KF, Pace RD, Mahboob M and Khan AT. 2008. Effects of dietary vitamin E, C and soybean oil supplementation on antioxidant enzyme activities in liver and muscles of rats. *Food Chem. Toxicol*., **46**: 3290–3294.

Singh VK, Dixit P and Saxena PN. 2005. Cybil induced hepatobiochemical changes in wistar rats. *J Environ Biol.,* **26(4):**725–727.

Tarladacalisir YT, Uygun M, Akpolat M and Hulya UZ. 2005. Histologic examination of the protective effect of vitamin E and C in cisplatin- induced hepatotoxicity. *Trakya Univ. Fak. Derg*. **22**: 124-131.

TayalaV,Kalraa BS, Agarwal SB, Khuran NA and Guptaa U. 2007. Hepatoprotective effect oftocopherol against isoniazid and rifampicin induced hepatotoxicity in albino rabbits. *Ind J Exp Biol*., **45**: 1031-1036.

Ukpanukpong RU, Eteng MU and Dasofunj OK. 2013. Antioxidant interactions of Pefloxacin, garlic, vitamins C and E on lipid profile level of albino wistar rats. *J App Pharm Sci*., **3 (3):** 167-170.

Velanganni AJ and Balasundaram C. 2003. Protective effect of vitamin A, ascorbic acid and α-tocopherol on 2,4-dimethylaminoazobenzene-induced hepatoma in rats. *Current. Sci*., **85**: 201- 203.

Westphal JF, Vetter D and Brogard JM. 1994. Hepatic side-effects of antibiotics. *J Antimicrob Chemother.,* **33**: 387–401.

JJBS

Volume 7, Number 2, June .2014
ISSN 1995-6673
Pages 133 - 137

Jordan Journal of Biological Sciences

Bioactive Crude Extracts from Four Bacterial Isolates of Marine Sediments from Red Sea, Gulf of Aqaba, Jordan

Wael A. Al-Zereini*

Department of Biological Sciences, Mutah UniversityP.O. BOX 7, Mutah 61710, Al-Karak–Jordan

Received: February 24, 2014 Revised: March 9, 2014 Accepted: March 13, 2014

Abstract

Escalation in number of resistant pathogens and diseases enhanced scientists to explore the unconventional habitats for bioactive compounds. Marine microorganisms represent a promising source for natural products due to the incredible diversity of chemical compounds that were isolated. Due to distinctive features and biodiversity of Red Sea biota, it was selected as a resource for isolation of bacteria with interesting bioactivity. Detection of bioactivity was performed using antimicrobial and antioxidant assays. Four bacterial isolates were found to have bioactivity at least in one assay. Crude extract of *Brevibacterium* sp. has weak antibacterial and moderate antioxidant effects, *Moraxella* sp. with weak antibacterial crude extract, and *Corynebacterium* sp. with potent antioxidant activity. Therefore, sediment marine bacteria represent an interesting source for antimicrobial and antioxidant secondary metabolites.

Keywords: Marine Bacteria, Antimicrobial, Antioxidant, Red Sea, Gulf of Aqaba.

1. Introduction

Despite the large number and diversity of bioactive compounds isolated from terrestrial microorganisms, since the penicillin era, new infectious diseases and resistant pathogens still represent a serious problem for human life (Cragg *et al.,* 1997; Desriac *et al.,* 2013). Therefore, the exploration of new and under-explored sources becomes extremely important in finding compounds with interesting bioactivities that can be used as new antibiotics (Penesyan *et al.,* 2011).

The oceans cover almost 70% of the earth's surface and contain a variety of species, many of which have no terrestrial counterparts (Whitehead, 1999). Marine bacteria as other marine biota produce novel compounds with unique structures (Boobathy *et al.,* 2009; Gram et al., 2010). They occupy different niches in the ocean, either planktonic, associated with inert or biotic surfaces, or they inhabit the sediments (Jayanth *et al.,* 2002). Approximately, 230 structurally characterized bioactive marine natural products were reported from 2009-2011, of which 102 compounds have antimicrobial activities (Mayer *et al.,* 2013).

The Red Sea has distinctive features such as unique coral reef systems, high level of available marine biota and great seasonal fluctuation of air and water temperature (Temraz *et al.,* 2006). Thus, it represents a valuable environmental source for organisms with promising bioactive metabolites. To date, few studies were performed on the Red Sea bacteria from the Jordanian side of the Gulf of Aqaba. They are represented by a publication on bioactivity of secondary metabolites isolated from a bacterium *Vibrio* sp. associated with the soft coral *Sinularia polydactyla* (Al-Zereini *et al.,* 2010), and master theses on bacterial communities associated with reef corals (Al Khateeb, 2011; Jaber, 2012; Khalfa, 2013).

In an ongoing research, the author is interested in screening natural crude extracts and isolation of biologically active compounds from marine organisms. Herein, isolation, cultivation, and extraction of antimicrobial and antioxidant crude extracts from four Red Sea sediment bacteria are described.

2. Materials and Methods

2.1. Collection of Marine Sediments

Sediment samples were collected in April 2008 in sterile plastic bags from different locations in front of the Marine Science Station (MSS)/Gulf of Aqaba (29°27 latitude and 34°58 longitude), at 10 m depth by SCUBA diving (Figure 1). Collection sites are characterized by presence of fringing reefs in some locations and sea grass meadows and sandy bottoms in other locations. These samples were transported in refrigerated containers (4 °C) to the laboratory in Mutah University.

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

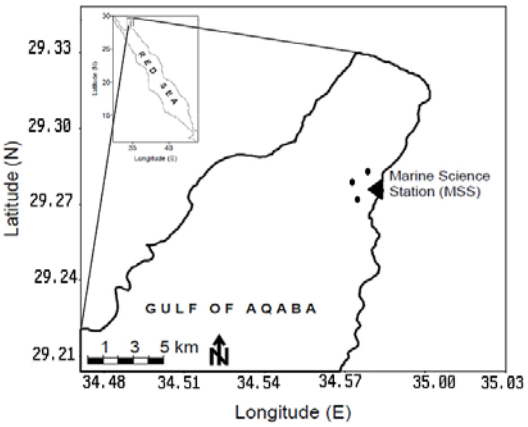

Figure 1. Collection sites in front of Marine Science Station on the Jordanian coast of Gulf of Aqaba.

2.2. Isolation, Characterization and Identification of Bacterial Samples

One gram of each sand sample was suspended in 10 ml sterile filtered sea water. The suspensions were then serially diluted (10^{-2} - 10^{-6}) with sterile filtered sea water. 50 µl from each tube was spread on modified Luria-Bertani (mLB) agar plates (0.5% tryptone, 0.5% yeast extract, 1% NaCl, 1.8% agar in a half strength marine sea water, pH 7.2) supplemented with cycloheximide (50 mg/l) and nystatin (50 mg/l) to inhibit the growth of yeasts and fungi. The plates were monitored for bacterial growth. New colonies were streaked on new agar plates for purification. This process was repeated several times till pure culture plates were obtained. 2 ml from culture of each pure bacteria isolate were conserved in 80% glycerol (1:1) and stored at – 20 °C till the date of screening.

Strains that show activity at least in one assay were identified using either RapID^{TM} ONE system (Remel, USA) and RapID^{TM} NF plus system (Remel, USA) for oxidase negative Gram negative bacteria, and oxidase positive Gram negative bacteria respectively or RapID^{TM} CB plus system (Remel, USA) for Gram positive bacteria.

2.3. Bacterial Cultivation and Extraction of Fluid Cultures

Bacterial strains were cultured in 1L Erlenmeyer flasks containing 500 ml of mLB medium on an orbital shaker (120 rpm, Forma Orbital Shaker, Thermo electron cooperation, USA) at 25 °C. During the fermentation process, 10 ml sample and thereafter daily samples were taken to monitor the bacterial growth. The growth was followed by OD measurements of 10 fold-diluted samples at 600 nm (UV/Vis Spectrometer, Lambda 16, Perkin-Elmer, Langen), and by changes in pH value (pH 523, WTW, Germany). As the OD ceased, the culture fluid was separated from the bacterial cells by centrifugation (5000g, 15 minutes, Beckman GS-6, Beckman coulter/USA). The supernatant was adjusted to pH 4 and extracted with an equal volume of ethyl acetate. The organic phase was dried over Na_2SO_4, concentrated *in vacuo* at 45 °C and the resulting residue was dissolved in methanol to a final concentration of 10 mg/ml.

2.4. In Vitro Antimicrobial Activity

The antibacterial activity was determined by agar diffusion test and the minimum inhibitory concentration (MIC) was determined by serial dilution assay according to the National Committee for Clinical Laboratory Standards (NCCLS, 2004) with some modifications. The test microorganisms used in this study were *Staphylococcus aureus* ATCC 43300, *Escherichia coli* ATCC 25922**,** *Klebsiella pneumoniae* ATCC 13883, and *Bacillus subtilis* ATCC 6633, seeded on LB agar plates (0.5% tryptone, 0.5% yeast extract, 1% NaCl, 1.8% agar).

Briefly, Muller-Hinton agar plates were seeded with 10^6 cell/ml of overnight grown test bacterial strains and were used for agar diffusion test. Six millimeter sterile filter paper discs impregnated with 300 µg/disc of the test samples were placed on the surface of the plates. All the plates were incubated at 37 °C for 24–48 hr. Antimicrobial activity was calculated by measuring the diameter of the inhibition zones. Minimum inhibitory concentration (MIC) was determined using serial dilutions of test samples and positive control (Chloramphenicol) starting from a final concentration of 1 mg/ml and 100 µg/ml, respectively. Test samples (100 µl) were diluted serially in 96 well plates and each microbial strain suspension (100 µl of 2×10^6 cell /ml) was added in each well. All prepared cultures were incubated at 37 °C for 24 hr. The MIC was determined as the minimum concentration of test sample that inhibits growth of microorganism and the OD_{600nm} of the culture is near to or equal zero. The experiment was performed in triplicate.

2.5. Antioxidant Assay

Antioxidant activity was measured in terms of the radical scavenging ability and decolorization of both used radicals, 2,20-azinobis(3-ethyl-benzothiazoline-6-sulfonic acid) (ABTS) and 1,1-Diphenyl-2-picrylhydrazyl (DPPH).

The antioxidant capacity assay was carried out using the improved $ABTS^{\bullet+}$ assay according to Re *et al*. (1999). Briefly, $ABTS^{\bullet+}$ radical cation was generated by reacting 7 mM ABTS and 2.45 mM potassium persulfate (final concentration in distilled water) and incubation at room temperature in darkness for 16 hr. The $ABTS^{\bullet+}$ solution was diluted with ethanol to an absorbance of 0.700 ± 0.005 at 734 nm UV/VIS Spectrophotometer. Crude extracts were concentrated in 20 mg/ml methanol, such that to give a maximum of 80% inhibition of the blank absorbance with 10 µl of sample. To 2 ml of diluted $ABTS^{\bullet+}$, 10 µl of each extract solution was added and mixed vigorously. The reactive mixture was allowed to stand at room temperature for 6 min and the absorbance was recorded immediately at 734 nm. Trolox standard solutions (concentrations from 0 to 20 µM) in ethanol were prepared and assayed using the same conditions. As blanks the same volume of solvents was run in each assay. The percentage of inhibition of absorbance at 734 nm was calculated and the results were expressed as a function of concentration of trolox for the standard reference data. Results were expressed in terms of trolox equivalent antioxidant capacity (TEAC), i.e., µM trolox/mg crude extract of coral or µM trolox/mg crude extract of bacteria culture. Assay was performed in

triplicate for each sample and each concentration of standard.

Scavenging ability of DPPH radical was estimated according to the method of Brand- Williams *et al*. (1995) with minor modification. 12.5 µl of different extracts was added to 2.5 ml methanolic solution of 0.1 mM DPPH. The mixture was shaken vigorously and left to stand at room temperature for 30 min. The decrease in absorbance of the resulting solution was measured at 517 nm by UV/Vis spectrophotometer. Methanol (99.5%) was used as a blank. Trolox standard (concentrations from 0 to 20 µM) in ethanol was prepared and assayed using the same conditions. The scavenging effect of the DPPH radical by the sample was calculated according to the formula

$$Scavenging\ effect\ (\%) = [(A_{control} - A_{sample})/ A_{control}] \times 100$$

Results were expressed in terms of mean value of trolox equivalent antioxidant capacity (TEAC). Assay was performed in triplicate for each sample and each concentration of standard.

2.6. *Statistical Analysis*

Means and standard deviations were deduced using *Excel* software.

3. Results and Discussion

In this work, twenty six bacterial isolates were obtained from collected samples. They were selected based on differences on morphological and biochemical tests. Twenty of these isolates (77%) are Gram positive bacteria while the Gram negative ones are represented by six isolates (23%).

During screening, two bacterial isolates showed activity in agar diffusion test, a Gram positive (*Brevibacterium* sp.) and Gram negative (*Moraxella* sp.) bacteria. They were isolated from sand samples in the sea grass area and their bioactivities against the tested microorganisms are summarized in tables 1 and 2.

Table 1. The inhibition zones (mm) caused by active bacterial isolates against tested microorganisms in agar diffusion test.

Test microorganism				
Name of isolates	***K. pneumoniae***	***E. coli***	***S. aureus***	***B. subtilis***
	Inhibition zone(mm ±SD[a]) 300 µg/disc			
Brevibacterium sp.	-	-	-	8 ± 0.6
Moraxella sp.	-	-	8 ± 0.6	-

[(a)] Standard deviation

The crude extracts of interesting isolates were weakly active against Gram positive test strains (*S. aureus* and *B. subtilis*), while Gram negative bacteria were resistant to the applied substances. This resistance could be attributed to low permeability of the cell wall of these strains to the bioactive ingredients in the extracts.

Table 2. Minimum inhibitory concentration (MIC) of the active bacterial crude extracts against susceptible microorganisms in serial dilution assay.

Name of isolates	**MIC (µg/ml)**	
	S. aureus	***B. subtilis***
Brevibacterium sp.	1000S[a]	500C
Moraxella sp.	500C[b]	1000S
Chloramphenicol	<8	<8

[(a)]: biostatic[(b)]: biocidal

To exclude the solubility problem of the extracts in agar, serial dilution assay was performed and the minimum inhibitory concentrations of the applied test substance were deduced. The results obtained in this test coincided with the agar diffusion test, where the extracts were weakly active against the susceptible Gram positive bacterial strains with MIC between 500-1000 µg/ml.

To date, *Brevibacterium* species are described as terrestrial representatives producing antimicrobial compounds only against Gram positive bacteria (Motta and Brandelli, 2002). Nevertheless, it was reported as a marine trait that inhibits Gram-negative pathogens, including *Klebsiella pneumoniae* and multi-drug resistant *E. coli* (Wietz, 2011). While secondary metabolites of *Moraxella* sp. was reported to exhibit various biological activity depending on medium and condition of bacterial cultivation (Nofiani *et al.*, 2012).

Moreover, *Brevibacteriun* sp. and *Corynebacterium* sp., isolated from the sand sample collected from sea grass area, were found to have antioxidant activity (Table 3) in ABTS assay. DPPH and ABTS procedures are commonly used in antioxidant activity assays in biological systems. They have a similar mechanism in that the absorption spectra of the stable free radical changes when the molecule is reduced by an antioxidant or a free radical species (Teow *et al.*, 2007).

ABTS assay was more predictive for antioxidant capacity than DPPH for extracts of marine bacteria. The $ABTS^{\bullet+}$ radical scavenging activity of 100 µg/ml of bacterial crude extracts ranged between 54%-63% (TEAC 119-138 µM Trolox/mg crude extract) with *Corynebacterium* sp. produce metabolites with most potent antioxidant activity. The higher values obtained in ABTS compared to DPPH could be attribute to the fact that ABTS detect antioxidant capacity of hydrophobic and hydrophilic extracts while DPPH predict antioxidant capacity for hydrophobic antioxidants. Similar observation was noticed by Rivero-pérze and his colleague in the antioxidant profile of red wine (Rivero-pérze *et al.*, 2007). In addition, ABTS is soluble in both aqueous and organic solvents and reacts relatively rapidly compared to DPPH.

Table 3. The antioxidant capacity of bacterial crude extracts measured as DPPH and ABTS. TEAC (µM Trolox/mg crude extract)

Name of isolates	DPPH (±SD[a])		ABTS (±SD)	
	TEAC	% inhibition	TEAC	% inhibition
Brevibacterium sp.	124.9 (±51.2)	32.29 (±13.8)	119.14 (±29.1)	54.22 (±13.66)
Corynebacterium sp.	115.73 (±20.75)	29.82 (±5.61)	138.31 (±6.09)	63.29 (±2.86)

(a)Standard deviation

The antimicrobial activity has been extensively reported for extracts of various groups of marine organisms (Blunt *et al.*, 2012 and the previous reports in this series). Higher percentages of microbial biologically active compounds were isolated from symbiotic bacteria associated with marine macroorganisms than from free living bacteria (Shnit-Orland and Kushmaro 2008). Recently, numerous compounds were isolated from Red Sea bacteria with antimicrobial as well as cytotoxic activities (Al-Zereini *et al*., 2010; Shaaban *et al*., 2013).

Nowadays, there is a great interest in evaluating the protective activity of natural antioxidants. Marine bacteria provided a resource of novel antioxidants with potential application in biomedicine, in food and feed, and in cosmetics or related products (Dunlap *et al.*, 2003). Bacterial species associated with seaweed from the Red Sea were found to give extracts with higher scavenging effect on DPPH radical (Abdel-Wahab *et al.*, 2013). Microorganisms, especially in marine niches where there is high reactive oxygen species (ROS) at electron rich areas due to metabolic and photosynthetic activity, are exposed to high level of oxidative stress through a combination of photosynthesis, symbiont oxygen production, and intense sunlight intensities leading to UV-induced free radical production (Townsend, 2008). Thus, as a protective mechanism, they produce metabolites that reduce the effect of the resulting oxidative radicals.

4. Conclusion

Marine sediment bacteria are promising sources for biologically active metabolites. In the present study, the crude extracts of interesting bacterial isolates exhibited antimicrobial and antioxidant activities. Therefore, further studies are required to isolate and purify the individual compounds behind such activities and elucidate their structures.

Acknowledgment

This project was financially supported by the Deanship of Scientific Research/Mutah University (No. 120/14/432). The author would like to thank Mr. Osama Al-Odat for his technical assistance.

References

Abdel-Wahab N, Ahmed E, Taie H, Hassan H, Abdel Hameed M and Hammouda O. 2013. Investigation of the antioxidant activity of some marine bacteria associated with some seaweeds from Red Sea. *New York Science Journal*., **6**: 27-32.

Al Khateeb S. 2011. Identification and charactyerization of bacterial community associated with coral reef in the Gulf of aqaba in Jordan. MSc thesis, Yarmouk University, Irbid, Jordan.

Al-Zereini W, Fotso Fondja Yao C, Laatsch H and Anke H. 2010. Aqabamycins A-G: novel nitro maleimides from a marine *Vibrio* species. I. Taxonomy, fermentation, isolation and biological activities. *J. Antibiot.* **63**: 297-301

Blunt J, Copp B, Keyzers R, Munro M and Prinsep M. 2012. Marine natural products. *Nat Prod Rep.*, **29**: 144-222.

Boobathy S, Kumar T and Katherisan K. 2009. Isolation of symbiotic bacteriaand bioactive proteins from the marine sponge *Callyspongia diffusa. Indian J Biotechnol.*, **8**: 272-275.

Brand-Williams W, Cuvelier M and Berset C. 1995. Use of free radical method to evaluate antioxidant activity. *Food Sci Technol.*, **28**:25-30.

Cragg GM, Newman DJ and Snader, KM. 1997. Natural products in drug discovery and development. *J Nat Prod.*, **60**: 52-60.

Desriac F, Jégou C, Balnois E, Brillet B, Le Chevalier P and Fleury Y. 2013. Antimicrobial peptides from marine proteobacteria. *Mar Drugs.*, **11**: 3632-3660.

Dunlap W, Llewellyn W, Doyle J and Yamamoto Y. 2003. A microtiter plate assay for screening antioxidant activity in extracts of marine organisms. *Mar Biotechnol*., **5**: 294–301.

Gram L, Melchiorsen J and Bruhn J. 2010. Antibacterial activity of marine culturable bacteria collected from a global sampling of ocean surface waters and surface swabs of marine organisms. *Mar Biotechnol.*, **12**:439–451.

Jaber F. 2012. Identification, characterization and seasonality of bacterial communities associated with coral reefs in the Gulf of Aqaba in Jordan. MSc thesis, Yarmouk University, Irbid, Jordan.

Jayanth K, Jeyasekaran G and Jeya Shakila, R. 2002. Isolation of marine bacteria, antagonistic to human pathogens. *Ind J Mar Sci*., **31**: 39-44.

Khalfa H. 2013. Extraction and partial characterization of antimicrobial agents produced by *Stylophora pistillata* and *Pocillopora damicornis* associated bacteria, from Gulf of aqaba, Jordan. MSc thesis, Yarmouk University, Irbid, Jordan.

Mayer A, Rodríguez A, Taglialatela-Scafati O and Fusetan A. 2013. Marine pharmacology in 2009–2011: marine compounds with antibacterial, antidiabetic, antifungal, anti-inflammatory, antiprotozoal, antituberculosis, and antiviral activities; affecting the immune and nervous systems, and other miscellaneous mechanisms of action. *Mar Drugs.*, **11**: 2510-2573.

Motta A and Brandelli A. 2002. Characterization of an antibacterial peptide produced by *Brevibacterium linens*. *J Appl Microbiol*., **92**:63-70.

National Committee for Clinical Laboratory Standards (NCCLS). 2004. Performance standards for antimicrobial susceptibility testing; fourteenth informational supplement NCCLS documents M100-S14. NCCLS, Wayne, Pennsylvania.

Nofiani R, Kurniawan R, Mabella T, Ardiningsih P, Zahara T and Sunarto. 2012. Study of media effect toward antimicrobial, antiplasmodial, herbicidal activities and toxicity of extract produced by *Acropora dibranchiata*-associated *Moraxella* sp. RA15. *J Appl Pharmaceut Sci*., **2**: 10-14.

Penesyan A, Tebben j, Lee M, Thomas T, Kjelleberg S, Harder T and Egan S. 2011. Identification of the antibacterial compound produced by the marine epiphytic bacterium *Pseudovibrio* sp. D323 and related sponge-associated bacteria. *Mar Drugs.*, **9**: 1391-1402.

Re R, Pellegrini N, Proteggente A, Pannala A, Yang M and Rice-Evans C. 1999. Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free Radic Biol Med.*, **26**: 1231–1237.

Rivero-Pérez M, Muñiz P and González-Sanjosé M. 2007. Antioxidant profile of red wines evaluated by total antioxidant capacity, scavenger activity, and biomarkers of oxidative stress methodologies. *J Agric Food Chem.*, **55**: 5476–5483.

Shaaban M, Abdel-Razik A, Abdel-Aziz M, AbouZied A and Fadel M. 2013. Bioactive secondary metabolites from marine *Streptomyces albogriseolus* isolated from Red Sea Coast. *J Appl Sci Res.*, **9**: 996-1003.

Shnit-Orland M and Kushmaro A. 2008. Coral mucus bacteria as a source for antibacterial activity. *Proceedings of the 11th international Coral Reef Symposium.* Fort Lauderdale. Florida, USA., **1**: 261-263.

Temraz T, Houssen W, Jaspars M, Woolley D, Wease K, Davies S and Scott R. 2006. A pyridinium derivative from Red Sea soft corals inhibited voltage-activated potassium conductances and increased excitability of rat cultured sensory neurons. *BMC Pharmacology.*, **6**: 10. (*Doi:10.1186/1471-2210-6-10*).

Teow C, Truong V, McFeeters R, Thompson R, Pecota K and Yencho G. 2007. Antioxidant activities, phenolic and b-carotene contents of sweet potato genotypes with varying flesh colours. *Food Chemistry.*, **103**: 829–838

Townsend B. 2008. Identification of marine antioxidants. MSc thesis, University of Georgia, Athens, Georgia.

Whitehead R. 1999. Natural product chemistry. *Annu Rep Prog Chem Sec B.*, **95**: 183-205.

Wietz A. 2011. Global patterns of marine bacterioplankton diversity and characterization of bioactive *Vibrionaceae* isolates. PhD thesis, Technical University of Denmark, Lyngby, Denmark.

JJBS

Volume 7, Number 2, June .2014
ISSN 1995-6673
Pages 139 - 145

Jordan Journal of Biological Sciences

Determination of Minimum Inhibitory Concentration of Cycloserine in Multidrug Resistant *Mycobacterium tuberculosis* Isolates

Rishan Singh*

University of KwaZulu-Natal, Westville, Durban, 4001, Republic of South Africa

Received: February 26, 2014 *Revised: March 12, 2014* *Accepted: March 21, 2014*

Abstract

This study was performed to determine the minimum inhibitory concentration (MIC) of cycloserine on 48 multidrug resistant tuberculosis (MDR-TB) isolates using the Broth Microdilution Method. No relationship between specific concentrations of cycloserine administrated and the bacterial inoculums used was found. This implied that the clinical isolates were obtained from patients on different treatment regimens. The findings of this study would potentially be important in alleviating the toxic effects of cycloserine in MDR-TB-infected patients while attempting to maintain effective doses.

Keywords: *: Cycloserine, Tuberculosis, Susceptibility, Resistance, Colonies.*

1. Introduction

Mycobacterium tuberculosis (TB) is the etiological agent of tuberculosis in humans. *M. tuberculosis* is a large non-motile rod-shaped bacterium that has a slow generation time (of between 15 and 20 hours), and this contributes to its invasive and aggressive spread in the well-aerated upper lobes of the lungs (Todar, 2011). Patients tend to become drug resistant to tuberculosis drugs or agents when the bacterium mutates (Singh, 2013). In addition, lipids (Mycolic acids), cord factor, and wax-D, that constitute the mycobacterial cell wall, facilitate development of resistance to various drugs (Todar, 2011). Many drug compounds are unable to enter the cell surface of mycobacteria because of this alpha-branched hydrophobic lipid layer, which contributes to its virulence. As a result, this has prompted the design of drugs that can cross this lipid barrier leading to death of the bacterium and allowing it to be destroyed by macrophages (instead of allowing their survival in macrophages as facultative intracellular parasites) (Todar, 2011; Singh, 2013).

Performing and interpreting minimum inhibitory concentrations (MICs) of TB isolates is important to prevent their spread across nations, and particularly in developing countries, where people are living with different stages of this disease and are given different types of treatment regimens. Tuberculosis has the most impact in developing countries because of resource limitations and the low socioeconomic living standards (Singh, 2013).

For this study, the MIC was defined as the lowest concentration at which cycloserine, a second-line, anti-tuberculosis drug, had to be administered in order to hinder the multiplication of tuberculosis isolates. In this study, Multidrug Resistant (MDR)-TB isolates were treated with this drug. Cycloserine ($C_3H_6N_2O_2$ (Sigma, 2008)) is a broad-spectrum antibiotic that is bacteriostatic at the recommended dosage. It is usually purchased either in capsule form or as a whitish/white-yellow powder that can be dissolved completely in water or partially in ethanol (Official Monographs for Part 1). Its usage has become limited particularly because of its hypersensitivity- (WHO PAR Part 4, 2007) and neurologically-related complications (Wolinsky, 1993) that have made the treatment of patients too costly. These costs arise from patients becoming obliged to monthly neuropsychiatric assessments. Some of the neuropsychiatric complications for which patients are treated include: confusion, convulsions, depression, dysarthria, headache, paresis, psychosis, somnolence, tremor, vertigo and the less well-understood human pregnancy/mother breastfeeding cases on treatment (WHO PAR Part 4, 2007). However, in spite of these complications, cycloserine has been used for renal and hepatic treatment in patients (Singh, 2012).

This study was therefore undertaken to determine the MIC of 48 MDR-TB isolates through treatment with five different concentrations of cycloserine (8, 16, 32, 64, >64 µg/mL). Thus, this study is a crucial step in alleviating the toxic effect of cycloserine in MDR-TB-infected patients, while attempting to maintain effective concentrations in the serum of infected patients.

* Corresponding author. e-mail: rshnsingh1@yahoo.com,Singhr6@ukzn.ac.za.

2. Materials and Methods

2.1. Bacterial Strains

Multidrug resistant tuberculosis (MDR-TB) isolates were frozen in cryovials using 0.5 mL freezing solution that contained 2g protease peptone and 16 mL glycerol. These isolates were retrieved from a reputable hospital in the KwaZulu-Natal area in South Africa. Since this study reports clinical isolates, the experimental names of the isolates have been withheld for confidentiality purposes. The experimental numbers start with the letter 'E' as indicated in the Appendix. $H_{37}R_v$, a susceptible tuberculosis isolate, was used as the control strain in this study because of its capability to utilise drugs efficiently compared to drug-resistant TB isolates. Therefore it was used as a reference when interpreting cycloserine susceptibility results in this study.

Appendix

Table 1. Cycloserine MIC results for clinical and control TB isolates at pH 7.2.

Number	Experiment no.	Day 7	Day 14	Day 21 (MIC)	Day 28	Inoculum (CFU/mL)
1.	E.12.	32	64	64	>64	7×10^6
2.	E.18.	32	32	64	64	4×10^6
3.	E.22.	32	32	64	>64	7×10^6
4.	E.23.	16	32	64	64	6×10^6
A.	$H_{37}R_v$	16	32	32	64	1×10^6
5.	E.25.	8	32	32	32	5×10^6
6.	E.28.	16	32	32	32	8×10^6
7.	E.30.	16	16	32	32	6×10^6
B.	$H_{37}R_v$	8	32	32	32	5×10^6
8.	E.33.	16	16	32	64	7×10^6
9.	E.35.	16	16	16	64	7×10^6
10.	E.39.	16	32	32	64	7×10^6
11.	E.51.	16	16	32	64	1×10^7
12.	E.29.	16	16	32	64	7×10^6
C.	$H_{37}R_v$	8	16	32	32	5×10^6
13.	E.40.	16	32	64	>64	7×10^6
14.	E.15.	>64	>64	>64	64	9×10^6
15.	E.47.	>64	>64	>64	64	8×10^6
D.	$H_{37}R_v$	16	32	64	64	5×10^6
16.	E.31.	32	32	64	64	9×10^6
17.	E.41.	32	32	64	64	7×10^6
18.	E.53.	32	64	>64	>64	7×10^6
E.	$H_{37}R_v$	16	32	64	64	4×10^6
19.	E.36.	16	32	32	64	1×10^7
20.	E.70.	16	32	32	64	1×10^7
21.	E.54.	16	*	64	64	1×10^7
F.	$H_{37}R_v$	8	16	32	32	4×10^6
22.	E.24.	32	32	64	64	7×10^6
23.	E.26.	32	32	64	64	2×10^7
24.	E.14.	16	32	64	64	7×10^6
G.	$H_{37}R_v$	8	16	32	64	8×10^6
25.	E.55.	16	32	32	64	1×10^7
26.	E.60.	16	32	32	64	1×10^7
27.	E.66.	16	32	64	64	2×10^6
28.	E.58.	16	32	64	64	2×10^7
29.	E.10.	16	32	32	64	1×10^7
H.	$H_{37}R_v$	16	16	32	64	1×10^7
30.	E.65.	16	32	64	64	8×10^6
31	E.24.	16	32	64	64	9×10^6
32.	E.71.	64	64	>64	>64	7×10^6
I.	$H_{37}R_v$	16	32	64	64	8×10^6
33.	E.63.	16	*	64	64	7×10^6
34.	E.67.	16	*	64	64	7×10^6
35.	E.74.	16	32	64	64	7×10^6
36.	E.80.	64	64	>64	>64	7×10^6
37.	E.83.	16	64	>64	>64	7×10^6
J.	$H_{37}R_v$	8	16	32	64	8×10^6
38.	E.81.	16	32	64	64	4×10^6
39.	E.82.	8	16	32	64	7×10^6
40.	E.9.	16	32	32	64	7×10^6
K.	$H_{37}R_v$	8	16	32	64	8×10^6
41.	E.79.	16	32	32	64	8×10^6
42.	E.8.	16	32	32	64	5×10^6
43.	E.7.	16	32	32	*	7×10^6
44.	E.6.	64	64	>64	>64	5×10^6
L.	$H_{37}R_v$	16	32	32	>64	4×10^5
45.	E.5.	16	16	32	64	7×10^6
46.	E.2.	16	16	32	64	7×10^6
47.	E.85.	16	16	32	64	7×10^6
48.	E.84.	16	32	32	64	7×10^6
M.	$H_{37}R_v$	8	16	32	32	5×10^6

Experiments were carried out in triplicates.

* indicates atypical or uncharacteristic TB susceptibility pattern.

2.2. Cycloserine

Cycloserine (product number C 6880 – 1 G) was purchased directly from Sigma-Aldrich Quimica, S.A. (Steinheim Germany). Aqueous solutions (pH 10) were prepared on the day of performing MIC experiments using the method recommended by Sigma (2008). The protocol involved making a 100 X concentrated 64 µg/mL cycloserine stock solution by dissolving the required amount in sodium carbonate (Na_2CO_3) solution (pH 10).

The cycloserine solution was subsequently filter-sterilised using a 0.22 µm sterile bell filter (Sarstedst, Numbrecht, Germany).

2.3. MIC Determination

Isolates were recovered by growing in Middlebrook 7H9 broth. In order to obtain the logarithmic growth phase, cultures were transferred to Middlebrook 7H11 at 37 ^{o}C. When the cultures reached the third week of growth, they were suspended in individual tubes containing 4.5 mL phosphate buffered saline (PBS), 0.05 % Tween 80 and 4 – 6 glass beads. The tubes were vortexed for 5 minutes and allowed to settle for approximately 45 minutes. Following this, the upper supernatant was aspirated and adjusted to a McFarland standard of 1 (10^7 colony forming units/mL) turbidity/density (National Committee for Clinical Laboratory Standard, 2002) by using sterile triple distilled water. Colony counts were performed on the respective isolates to determine the CFU/mL.

The broth micro-dilution procedure was performed in 24-well tissue culture plates. Middlebrook 7H9 broth (supplemented with 10% oleic acid-albumin-dextrose-catalase (OADC)) was adjusted to pH 7.2. Next, the three 64 µg/mL-labelled wells on each of the plates, were treated with 1800 µL of broth and 20 µL of cycloserine. Wells that contained 64 µg/ml of cycloserine (in triplicates) were two-fold diluted down to 1 µg/mL. Plate wells without cycloserine served as drug-free controls.

Control strain $H_{37}R_v$ was used whenever an isolate or a set of isolates were being tested. An inoculum of 100 µL of 10^5 CFU/mL of TB culture was used for each well of the plate (please note that the inoculum shown in the appendix represents the CFU/mL after cycloserine had been administrated and the plates were incubated for 1 – 4 weeks). The plates were incubated for 28 days. Wells were examined at 7, 14, 21 and 28 days in order to determine TB isolates' susceptibility trends. The MIC was determined after 21 days. The MIC of cycloserine, in this study, was defined as the lowest concentration which completely inhibits the growth of MDR-TB isolates at a pH of 7.2. This definition was used to interpret susceptibility results.

2.4. Calculation of Colony Counts

Colony counts were calculated for the 10^{-3} (dilution factor) plate cultures. This was performed to determine the number of colonies present at this dilution of culture (i.e., the number of CFU/mL after establishment of the growth logarithmic phase).

3. Results and Discussion

Mycobacterium tuberculosis-infected patients are treated with first-, second-, and third-line TB drugs. However, to optimise the dosage of the drugs in regard to toxicity of specific drugs given, TB-infected patients are put on combination therapies so that the high toxicity of drugs in the serum of patients is minimised. Singh (2013) reported a case in which TB drugs can become unstable in the serum of patients, and in the case of cycloserine-treated patients, combination therapies fail to apprehend the onset of adverse effects associated with the nervous system in particular. In addition, Wolinsky (1993) confirmed that cycloserine can potentiate hypersensitivity complications in TB-infected patients.

In this study, 48 MDR-TB isolates were treated with 5 different cycloserine concentrations (8, 16, 32, 64, and > 64 µg/mL). Cycloserine minimum inhibitory concentration was determined in a 7H9 broth-based system by monitoring TB growth in 7 day intervals (7, 14, 21, 28). The response of TB isolates to the different concentrations of cycloserine was monitored relative to the control strain, $H_{37}R_v$, and the susceptibility patterns and differences among the different sets of isolates tested on different days were noted and reported. Only one isolate [E.35.] out of the 48 tested isolates had an MIC of 16 µg/mL on the 21st day and this MIC did not correlate with the MIC of the control strain (32 µg/mL). This difference was significant as it indicated that the experiments were successfully standardised relative to susceptible control strain isolate. Similarly 74 % ($^{14}/_{19}$) of the isolates that were static at an MIC of 64 µg/mL of cycloserine, did not correlate with the MIC of the $H_{37}R_v$ strain (32 µg/mL). Those 14 isolates are presented in Table 1 (E.12., 18, 22, 23, 54, 24, 26, 14, 66, 58, 63, 58, 63, 67, 74, 81). The remaining 5 isolates ($^{5}/_{19}$) were inhibited at 64 µg/mL of cycloserine in conjunction with their respectively tested $H_{37}R_v$ strains per reported sets. These isolates were: E.40., 31, 41, 65 and 24. Among 15 % ($^{7}/_{48}$) of MDR-TB isolates that were inhibited by cycloserine at a concentration of more than 64 µg/mL, all had MICs that did not correlate with the MIC of $H_{37}R_v$, but with a greater variability in the MIC of $H_{37}R_v$ in each set of tested MDR-TB isolates. Isolates E.15., 47, 53, and 71 exhibited a static effect in multiplication when they were exposed to a cycloserine concentration of greater than 64 µg/ml, while the $H_{37}R_v$ strain was static at a reported MIC of 64 µg/mL. In contrast, $H_{37}R_v$ was inhibited at a concentration of 32 µg/mL, while cycloserine was bacteriostatic to isolates E.80, 83, and 6 at >64 µg/mL. The growth of the 21 isolates that had been inhibited by a cycloserine concentration of 32 µg/mL was in correlation with the MIC of $H_{37}R_v$ on their respective testing days, and in their tested sets. These isolates are also shown in Table 1: E.25., 28, 30, 33, 39, 51, 29, 36, 70, 55, 60, 10, 8, 2, 9, 79, 8, 7, 5, 2, 85, 84.

Table 1. Minimum Inhibitory Concentration (MIC) results of cycloserine on 48 MDR-TB isolates compared to their respective MIC results of the control, $H_{37}R_v$ on day 21.

MIC (µg/mL) (day 21)	Total no. of isolates	%	Isolates correlating with the MIC of $H_{37}R_v$	%	Isolates not correlating with the MIC of $H_{37}R_v$	%
8	0	0	n/a	n/a	n/a	n/a
16	1	2	0	0	[35]	2
32	21	48	[25, 28, 30]; [33, 39, 51, 29]; [36,70]; [55, 60, 10]; [55, 60, 10]; [82, 9]; [79, 8, 7*]; [5, 2, 85,84]	100	0	0
64	19	40	[40]; [31,41]; [65, 24]	26	[12, 18, 22, 23]; [54]; [24, 26, 14]; [66, 58]; [63, 67, 74]; [81]	74
>64	7	15	0	0	[15, 47]; [53]; [71]; [80, 83]; [6]	100

*atypical growth pattern

n/a: not applicable

Brackets [ ] denote isolates tested on the same day.

Of the 21 conforming MDR-TB isolates, isolate E.7 exhibited an atypical susceptibility pattern after it had been exposed to cycloserine. It was classified as atypical because on the day of reading the MIC, growth was observed in the wells that contained 16 µg/mL and 64 µg/mL of cycloserine. Furthermore, this isolate had been susceptible to the same concentration of cycloserine (32 µg/mL) after 14 days of incubation (as the MIC read date). However, the MIC reading was invalid due to finding growth in the tissue-culture plate after 21 days. This was not ascribed to inappropriate colony counts as reported in Singh (2013) because other MDR-TB isolates had valid cycloserine MIC readings with the same colony count number as this isolate (7×10^6 CFU/mL). Those isolates, including $H_{37}R_v$ that were positively inhibited by cycloserine with a CFU/mL of 7×10^6 are: E.22., 33, 35, 39, 29, 40, 41, 17, 53, 24, 14, 71, 63, 67, 74, 80, 83, 82, 9, 5, 2, 85 and 84. This atypical result was not attributed to any pipetting errors of cycloserine into the tissue-culture plate because all of the MIC results were noted down in triplicates (Singh, 2013) as presented in Table 1. Petrini and Hoffner (1999) and Singh (2012) provide a possible explanation to such a result. These authors suggested that by genotypically testing this isolate, one may obtain an idea into its genetic profile since resistance-acquisition in tuberculosis is not attributed to plasmid insertion of resistance genes like in *Haemophilus ducreyi*, for example, but due to genetic mutations that are induced by the toxicity of the administered tuberculosis drugs (or agents) (Petrini and Hoffner, 1999; Singh, 2012, 2013). Therefore, such clinical isolates can possibly have increased virulence and result in increased TB spread, infection and disease to others (Singh, 2013). From Table 2, it is evident that this study presents many different scenarios for reporting and interpreting minimum inhibitory concentration results in relation to MDR-TB clinical isolates. This table presents uncharacteristic isolates (E.21, 63 and 67), apart from the atypical isolate E.7, and these isolates highlight the lack of confidence that one might get from relying on MIC values obtained on day 21. Since those isolates showed increased growth by the second week of the experiment, the MIC of cycloserine at day 21 (64 µg/mL) cannot be reported with reliability. Although the fourth week MIC reading was akin to the third week reading, it is uncertain whether the second week reading could have been the same. It's for that reason that this study reported these readings as MICs for the mentioned isolates. Pipetting errors and inappropriate colony counts are not attributable to these uncharacteristic results as previously discussed and mentioned in Singh (2012, 2013).

Table 2. Isolates showing atypical and uncharacteristic TB growth inhibition in the MIC study

Isolate Number	Minimum Inhibitory Concentration (MIC)				Inoculum CFU/mL
	Day 7	Day 14	Day 21	Day 28	
21	16	*	64	64	1×10^6
63	16	*	64	64	7×10^6
67	16	*	64	64	7×10^6
7	16	32	32	*	7×10^6

* indicates atypical or uncharacteristic growth pattern.

The MDR-TB isolates in this study were tested in sets and the MIC of cycloserine for each isolate in the set was reported relative to $H_{37}R_v$. Although isolate sets were tested for susceptibility to cycloserine, the MIC results obtained for all 48 isolates did not have much variability. This, however, is not acceptable since each MDR-TB-infected patient would have been on a different treatment regimen. Therefore as stressed in Singh (2013), the complete treatment profile of patients require assessment in order to accurately interpret the clinical isolates' cycloserine susceptibility patterns. Fourteen out of the 48 isolates (29 %; E.12., 28, 39, 41, 36, 70, 55, 60, 10, 9, 79, 8, 7, 84) showed a single shift in their MIC susceptibility pattern for cycloserine from day 7 to 14 to 21, while the remaining 71 % had susceptibility patterns whereby the MIC value were repeated (at least) two times across the four weeks. The latter includes isolate E.7, which was classified as being atypical. Of these 14 isolates, only 2 isolates (E.28 and 41) had day 28 susceptibility readings that correlated with day 21 MIC values. Both of these isolates were present in the broth-based system at $8 \times 10^6 - 7 \times 10^6$ CFU/mL. However, it was deduced that when MDR-TB isolates were present at a concentration of between $8 \times 10^6 - 1 \times 10^7$ CFU/mL in the broth-based system, the concentration of cycloserine that induced tuberculosis growth inhibition was 64 µg/ml or more, with the exceptions of isolates E.66, 81, 68, and 6, because those isolates were present below this colony

count number in the experiments and would not have been considered ideal according to the McFarland standard that was used (Singh, 2012).

MDR-TB isolates E.66 and 81 were inhibited by cycloserine at concentration of 64 µg/ml though the inoculums used were low (2×10^6 and 4×10^6 CFU/mL, respectively). In contrast, although isolate E.6 was inoculated at 5×10^6 CFU/mL, a far greater concentration of cycloserine was required to inhibit its multiplication (>64 µg/mL). Isolates E.12, 39, 41, 9 and 7 were inoculated at 7×10^6 CFU/mL, but bacterial growth was inhibited at different cycloserine concentrations. Isolates E.12 and 41 were inhibited at a cycloserine concentration of more than 64 µg/mL, while the remaining 3 isolates were inhibited at 32 µg/mL. Isolates E.36, 70, 55, 60, and 10 exhibited the same cycloserine susceptibility pattern across the four weeks and were inoculated into 7H9 at 1×10^7 CFU/mL. Although this was considered a significant discovery, other isolates (E.28., 39, 9, 79, 8, 84) were found to have the same cycloserine susceptibility pattern, but used different mycobacterium inoculum counts. Singh (2012, 2013) reported and commented on this finding and has raised the question of 'at what CFU/mL would performing MIC tests be optimal at, if such an optimal exists?' The MIC results for isolates E.36, 70, 55, 60, and 10, further reiterates the question put forth by Singh (2012, 2013).

Cell counting was used to optimise and standardise the protocols used in performing drug susceptibility tests, such as the determination of (MIC) values, by providing uniform colony counts amongst multiple experiments (Singh, 2013). However, with the patients being on different treatment regimens, optimising the susceptibility on the basis of colony counts alone is not sufficient, especially since the antibiogram of the isolates is not available, as is the case in the present study. Therefore, it's probable that conclusions from drug susceptibility tests cannot be sufficiently made by simply relying on CFU/mL as an indicator, because it is possible for some (or all) patients to be on combination therapies or utilising other forms of treatment options (Singh, 2012, 2013; personal writing, 2014).

In this study, although colony counts were important for measuring MICs and/or drug susceptibility tests, it was found that the inhibition of the bacterial multiplication machinery by various cycloserine concentrations, was not influenced by the amount of bacteria being used (reviewed in Singh, 2013). For example, $H_{37}R_v$ for isolates E.36, 70 and 54 showed an MIC of 32 µg/mL on day 21. The inoculum of the $H_{37}R_v$ strain for these 3 MDR-TB isolates tested was 4×10^6 CFU/mL (i.e. relatively low). It's possible that the persistence of the MIC value after 21 days was due to the low colony count numbers compared to the concentrations of $H_{37}R_v$ used in other experiments, except for isolates E.24., 14, and 66.

The $H_{37}R_v$ isolates that were used per set of MDR-TB isolates are shown in order of ascending inoculum counts in Table 3 (3×10^6 – 8×10^6 CFU/mL). Though a proportional relationship between TB colony forming units and the amount of cycloserine administered was expected, in this study, this relationship was not apparent because experiments involving similar $H_{37}R_v$ inoculums unexpectedly resulted in different cycloserine MIC values (see experiments involving sets B, C, D, and M). It was also found that the control strain $H_{37}R_v$ used in sets K, F, L and M, exhibited the same cycloserine susceptibility pattern across four weeks with a precise cycloserine MIC value being reached after the second week. The MICs of the control strain used in sets K, E, F, C, D, M and I, were considered feasible because the concentration of cycloserine that induced growth inhibition after 3 weeks was identical to that of the fourth week. These MIC readings increased the internal and external validity of the study as stated in Singh (2013). An exception was the control strain used in set B, because it had an MIC value identical to the reported cycloserine concentration after the first week of experimentation.

Table 3. $H_{37}R_v$ isolates with the same cycloserine inhibitor concentrations for days 21 and days 28. (arranged in ascending order of colony counts)

Inoculum CFU/mL	Control Strain Set	Day 7	Day 14	Day 21	Day 28
3×10^6	K	8	16	32	32
4×10^6	E	16	32	64	64
4×10^6	F	8	16	32	32
5×10^6	B	8	32	32	32
5×10^6	C	8	16	32	32
5×10^6	D	16	32	64	64
5×10^6	M	8	16	32	32
8×10^6	I	16	32	64	64

The McFarland standard is a turbidity standard used to measure out a particular concentration of microorganism required in an experimental study (Singh, 2013). It has been reported that a dilution factor of 10^4 (starting from 10^4) would provide a working concentration of 1×10^7 CFU/mL (Singh, 2013), but other studies used a dilution factor of 10^3. Control strain of sets K, F, C and M, exhibited the same susceptibility profiles, while those in sets E, D and I, exhibited the same susceptibility profiles, but at variable inoculum CFU/mL. Hence, although the inoculums for the controls strains in sets C, D, and M was 5×10^6 CFU/mL, the susceptibility pattern of the control strain in set D mimicked that of E and I, which had control strain inoculums of 7H9 at 4×10^6 and 8×10^6 CFU/mL respectively.

Researchers and others may find the MIC readings of the MDR-TB clinical isolates to be obscure when they are compared with that of the $H_{37}R_v$ strain, but as explained by Singh (2013), such MIC values are of value and of universal importance. Isolate E.6. was present in the wells of the tissue-culture plate at the same concentration as E and F. The susceptibility profiles of the 3 isolates showed a single shift in the cycloserine inhibitory potential, but the MIC at which the growth of each isolate was inhibited, was different. In conclusion, there was no established link between the established MIC readings and the colony counts of each isolate (Singh, 2012, 2013).

A significant finding of this study was that $H_{37}R_v$ isolates in sets A, L, and B (Table 4) had MIC readings

that did not strongly correlate with the isolate sets tested on their respective test days. Since the MIC readings of the mentioned isolates changed on the 28th day, those isolates probably required genotypic testing similar to what was suggested for the atypical isolate 7. Of these 3 isolates, isolate in set B exhibited the most accurate inhibitory growth effect against cycloserine, because it retained its cycloserine MIC value (32 µg/mL) after 21 days. In contrast, control strain in sets A and C required a much higher dosage of cycloserine to induce their inhibitory effect because these isolates did not retain their MIC values after the third week, even though the MIC value may or may not be identical after 14th day. The cycloserine MIC values of 64 and >64 µg/mL for control strains in sets A and L, indicated that the isolates were either too resistant to the administered concentrations, or they were not susceptible to the administered concentrations at that given point in time. Furthermore, no established links between the MDR-TB colony counts and the MIC patterns between these 3 isolates (A, L, and B) were found, and this is postulated to be true for all the isolates (including $H_{37}R_v$), that were tested. The control strain in sets C, D, E, F, G, H, I, J, K, L, M, and N, had well-established MIC results since the inhibitory cycloserine concentrations (day 21) were akin to the MIC reading after 3 weeks, and were different from the cycloserine inhibitory concentrations after 14 days.

Table 4. $H_{37}R_v$ isolates showing poor representation MIC results when administered with cycloserine between days 7 and 14 and days 14 and 21.

Inoculum CFU/mL	Key	Day 7	Day 14	Day 21	Day 28
1×10^6	A	16	32	32	64
4×10^6	L	16	32	32	>64
5×10^6	B	8	32	32	32

Due to the absence of information regarding the treatment regimens being used at the time the 48 MDR-TB isolates were obtained, the unusual MIC results of cycloserine on specific clinical isolates (such as E.7) cannot be explained with confidence (Singh, 2013). Furthermore, individual TB-infected patients often present themselves at different stages of tuberculosis infection for treatment, and may be falsely recognised as MDR-TB during drug susceptible laboratory tests (Singh, 2012, 2013). In the present study, this could have been the reason for the cycloserine susceptibility patterns obtained for isolates E.71., 80, 83, and 6. The colony forming units for E.6. was different compared to the other 3 tested isolates. Alexander and Strete (2001) has further suggested that MIC results this high (64 or >64 µg/mL) could be attributed to the immune-compromised state of the patient. Singh (2013) emphasises that this could have been due to late tuberculosis treatment and other allied problems like financial, economic, social and sexual reasons (the introverted state of the infectious disease). Furthermore, these patients could have also been shy to confront a physician, specialist or consultant because of additional problems like AIDS, HIV co-infection and other sexually transmitted diseases.

This study was also significant because many of the MDR-TB isolates were found to be susceptible to cycloserine at similar inhibitory concentrations for the first two weeks. Isolates E.18., 22, 23, 30, 33, 15, 51, 29, 15, 47, 31, 41, 31, 41, 24, 26, 71, 80, 6, 5, 2 and 85 exhibited this characteristic (Appendix). Although these MIC values did not conclude anything about the patient's resistance to cycloserine in comparison to the MIC values after 3 weeks, reporting them is an advantage as it confirms that the tested MDR-TB isolates had different susceptibilities to cycloserine. Moreover, the increase in MIC value after 14 days, indicated a mycobacterial shift of growth inhibition to a higher concentration of the drug (Singh, 2013).

Isolate E.83 (Appendix) had a very interesting susceptibility pattern to cycloserine across the four week susceptibility period. This isolate initially required 16 µg/mL of cycloserine to slow its multiplication down before one week exposure, and a 4-fold concentration by the 14th day. Singh (2013) suggests that this could have been due to an increased resistance of E.83. to cycloserine due to genetic mutations induced by exposure to cycloserine early in the experiment.

4. Conclusion

This study demonstrated the absence of a relationship between cycloserine MIC values of the TB isolates and the inoculum CFU/mL used. This study might serve as an important 'reference' to minimising the toxic effects of cycloserine so that it's prescription as a TB drug to patients can be safe and reliable, in that it will not have an effect on the nervous system and other hypersensitive complications. Generally a blood cycloserine concentration of 30 mg/L would cause these complications, but in this study, the concentration of cycloserine that caused growth inhibition of the MDR-TB isolates were variable and at a much lower concentration compared to this value. The results of this study would allow for future studies that focus on maintaining effective doses of cycloserine, while concurrently minimising its toxicity. The results of those studies would form an important step in the wider use of this important second-line TB drug.

References

Alexander SK and Strete D. 2001. **Microbiology: A Photographic Atlas For The Laboratory**, Benjamin Cummings, Addison Wesley Longman Inc, USA. pp. 127.

Official Monographs for Part 1. **Cyclophosphamide**, JP XIV. pp. 390-391 (year supplied).

Petrini B H S. 1999. Drug-resistant and multidrug-resistant tubercle bacilli. *Int J Antimicrobial Agents*. **13**: 93-97.

Sigma A. 2008. Cycloserine Product Information. Available from: www.sigma-aldrich.com. Accessed: 18 April 2008.

Singh R. 2013. Minimum inhibitory concentration: Interpretation and cross-Sectional analysis in an unstandarised 7H9 *Mycobacterium tuberculosis* broth-based system – A hypothetical case. *J Pure Appl Microbiol* **7(1)**: 777-784.

Todar. 2011. **Todar's Textbook Of Bacteriology: Tuberculosis**, Madison, Wisconson, USA. http://textbook/ bacteriology.net/tuberculosis.html. Accessed: 17 November 2011.

WHO PAR Part 4. 1997. Cycloserine Capsule 250 mg (Macleods) (Version 1.0.). TB154. pp-1-6.

Wolinsky E. 1993. Statement of the tuberculosis committee of the Infectious Disease Society of America **16**: 627-626.

JJBS

Jordan Journal of Biological Sciences

Volume 7, Number 2, June .2014
ISSN 1995-6673
Pages 147 - 151

Biochemical Parameters of Common Carp (*Cyprinus carpio*) Exposed to Crude Leaf Extract of *Cannabis sativa*

Bala S. Audu[1], Kabir M. Adamu[2,*] and Paul C. Ofojekwu[1]

[1]*Department of Zoology, University of Jos, P. M.B. 2084, Jos,*

[2]*Department of Biology, Ibrahim Badamasi Babangida University, P.M.B 11, Lapai, Niger State, Nigeria*

Received: March 11, 2014 Revised: April 18, 2014 Accepted: April 21, 2014

Abstract

The effect of sub-lethal concentrations (1.88, 3.75, 7.50, 15.00 and 30.00 mg/L) of crude leaf extract of *Cannabis sativa* was determined in the plasma, liver and gill biochemical parameters of Common carp, *Cyprinus carpio* (mean weight of 15.05±0.05g) after 56-day exposure period in static renewable bioassay system. During the experiment, some physico-chemical parameters were monitored while at the end of the experiment, the selected biochemical parameters were determined in the plasma, liver and gill of the test fish. The biochemical parameters determined were alkaline phosphatase (ALP), Lactate dehydrogenase (LDH), total protein (TP), aspartate aminotransferase (AST), alanine aminotransferase (ALT), bilirubin, total bilirubin (TB), albumin (ALB), urea acid (UA) and cholesterol (CHOL). The monitored pH showed significant difference (p<0.05) while water temperature, total alkalinity, dissolved oxygen and free carbon (iv) oxides showed no significant difference (p>0.05) in *C. sativa* test set-ups compared to the control during the experimental period. There were significant difference (p<0.05) in some of the determined biochemical parameters of the test fish exposed to *C. sativa* compared to the control. Therefore, it can be deduce from the study that prolonged exposure of *C. carpio* fingerlings to crude leaf extract of *C. sativa* affected some determined biochemical parameters in the tissue/organs of the test fish.

Keywords: *: Cannabis Sativa, Biochemical Parameters, Cyprinus Carpio .*

1. Introduction

Cannabis sativa is a cosmopolitan weedy plant that is grown in many parts of the world. It contains compounds with active ingredients of varying potencies such as tetrahydrocannabinol (THC) (Hampson *et al*., 2000; Koch, 2001), phytocannabinoids and plant steroids (Rifat, *et al.,* 2010; Audu *et al*., 2013). The phytochemical analysis of the leaves revealed the presence of alkaloid, flavonoids, cardiac glycosides, resins, terpenes and steroids (Audu *et al.,* 2013). Studies conducted by Amna (2011) revealed that *C. sativa* caused significance difference in certain clinical enzymes in rats and men. Studies have also noted the potency of cannabis as pest repellent and pesticides of potato beetle - *Leptinotarsa decemlineata* (Stratii, 1976), wheat root maggot-*Delia coarctata* (Pakhomov and Potushanskii, 1977) and root exudates of European chafer-*Melolontha melolontha* (Mateeva, 1995). The plant has been reported to have anesthesia effect on *Oreochromis niloticus* (Audu *et al.,* 2013).

Due to the fact that anaesthetics are used with increasing frequency in aquaculture, mainly to reduce the stress and to prevent mechanical damage to fish during handling as their use is particularly common in stripping, marking, health checks, etc (Ross and Ross 1999), there is the need to study their effect on the biochemical parameters of fish. The common carp (*Cyprinus carpio*) belongs to the family cyprinidae and is one of the most important breeder species (Mohamad *et al*., 2011) with an increasing farmers' preference. The fish is an economically significant species. It is cultivated commercially (Cao *et al.,* 2013) in other parts of the world including Austrialia and South America and Africa because of its fast growth rate, facile cultivation and high feed efficacy ratio (Tokur *et al.,* 2006). According to Jalali Mottahari *et al*. (2013) it is a potential species for fish culture since it can tolerate a wide range of changing water pH. The fish has gained researchers interest as test fish in conducting toxicity test with natural products (Selamoglu *et al*., 2012).

C. sativa have various applications with their attendant side effects, however, there is no scientific documentation of the effects of the crude leaf extract on the plasma, liver and gill biochemical parameters of *C. carpio*. Therefore, this study was undertaken to determine the effects of the crude leaf extract of *C. sativa* on the plasma, liver and gill biochemical parameters of *C. carpio* after the 56-day experiment in static renewable bioassay system.

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

2. Materials and Methods

Marijuana (*Cannabis sativa* (L.)) was obtained from the National Drug Law Enforcement Agency (NDLEA), Jos command, Plateau State, Nigeria, strictly for scientific research. The leaves were carefully sorted out from the stem/twigs by handpicking, ground into powder then sieved through a metal sieve (90µm mesh size) and stored in airtight polyethylene bag for use.

A stock solution of the crude leaf extracts was prepared by macerating 12.5g of the dried powdered leaves in 500ml of petroleum ether for 24 hours at 25^{o}C. From this oily (950mg petroleum ether free) resin, the concentrations used for the experiment were prepared. These concentrations were obtained from the value of 96 h LC50 as 30(I/3rd of LC50), 15 (1/6th of LC50), 7.50(1/12th of LC50), 3.75(1/24th of LC50) and 1.88mg/L (1/48th of LC50). These were placed into clean and dry conical flasks where 10, 20, 40, 80 and 100 ml of acetone was added respectively to give the appropriate concentrations used for the test. Water and acetone in water were used as control as described in UNEP (1989). A total of ten fish were distributed in each test concentration and controls in aquaria (60x40x40cm) with two replications.

Two hundred fingerlings of *C. carpio* average weight of 15.05 ±0.05g were obtained from the extension unit of Bauchi State Agricultural Development Project (BSADP) Bauchi State, Nigeria and transported to the Applied Hydrobiology and Fisheries Laboratory of University of Jos, Nigeria, in an oxygenated polyethylene bags. They were held in rectangular tanks and allowed to acclimatize for two weeks. During the acclimatization and exposure periods, the fish were fed to satiation with 3mm commercially pelleted fish feed (Multifeeds®; Protein 42%, Fat12%, Ash7.5% Fiber2.6%). Dechlorinated tap water was used during acclimatization and exposure periods. The experimental aquaria were supplied with continuous dissolved oxygen through a giant aeration pump. Physico- chemical parameters were monitored throughout the 56- day trial by the methods described in APHA *et al*. (1985). At the end of 56 day exposure period, the plasma, gills, and liver of exposed and controls fish were collected and examined for the level of alkaline phosphatase (ALP), lactate dehydrogenase (LDH) total protein (TP) albumin (ALB), aspartate aminotransferase (AST), alanine aminotransferase (ALT), bilirubin (BIL), total bilirubin (TBIL), uric acid (UA) and cholesterol (CHOL). Blood collection was through cardiac puncture using (1ml) non heparinised syringe and needle. The blood was immediately centrifuged at 1500 rpm for 5 minutes to obtain the plasma. The gills and liver samples were homogenized in buffer (0.25M sucrose, 0.01M TRIS and 0.01M EDTA) and centrifuged at 1500 rpm for 10 minutes where the supernatant was used for the analyses. The activities of ALP, LDH, TP, AST, ALT, BIL, TBIL, UA and CHOL were assayed with the aid of GENESYS-20 spectrophotometer (GENESYS 20.Thermo Electron Corporation USA) following the manufacturer's instruction of the of Randox, Spectrum and Fortress reagent kits.

Data obtained were analyzed for means, standard error, analysis of variance (ANOVA) and Duncan's multiple range tests for significance difference at 5% level of probability using the statistical package SPSS 17.0 computer program (SPSS Inc. Chicago, Illinois, USA).

3. Results

The monitored physico-chemical parameters of the experimental set-up are presented in Table 1. There were no significant difference (p>0.05) in all the monitored physico-chemical parameters except pH. As the concentrations of the C. sativa increased the test medium becomes acidic with the test medium (30.00 mg/L) recording the pH of 6.20 (0.45). There was no significant difference (p>0.05) recorded in the monitored physico-chemical parameters between the two control set-ups. The activities of ALP and LDH in the sampled tissue/organs of the test fish were presented in Table 2. As the concentrations of the plant leaf extract increased the activity of ALP and LDH in the plasma, gill and liver significantly decreased and increased (p<0.05) respectively after the 56-day experimental period. LDH and ALP activities in the control groups were observed to be highest in the gill, less in the liver and least in the plasma.

The activities of AST, ALT, ALB, TBIL, BIL, UA and CHOL in the plasma of the test fish exposed to the plant leaf extract were presented in Table 3. As the concentrations of the plant extract increased the activity of AST, ALT and UA increased while those of ALB, TBIL, BIL and CHOL were decreased. There was significant difference (p<0.05) in the plasma activities of ALT, UA and CHOL after the 56-day experimental period. The determined values of total proteins in plasma and liver of the test fish exposed to sublethal concentrations of the plant leaf extract were presented in Table 4. There were no significant difference (p>0.05) in the plasma and liver total protein level after the 56-day experimental period.

Table 1. Physico-chemical parameters of experimental set-up used to study the effect of *C. sativa* on the biochemical parameters of *C. carpio* during the 56-day experimental period

Physico-chemical parameters	**Concentration of *C. sativa* (mg/L)**						
	0.00 (Water)	**0.00 (Acetone)**	**1.88**	**3.75**	**7.50**	**15.00**	**30.00**
Temperature (°C)	22.40 (0.55)	22.40 (0.55)	22.40 (0.55)	22.40 (0.55)	22.40 (0.55)	22.40 (0.55)	22.40 (0.55)
pH	7.00 (0.00)	7.00 (0.00)	6.60 (0.31)	6.60 (0.22)	6.40 (0.41)	6.20 (0.45)*	6.20 (0.45)*
Dissolved oxygen (mg/l)	4.50 (0.00)	4.50 (0.00)	4.50 (0.00)	4.10 (0.22)	4.10 (0.22)	4.10 (0.22)	4.00 (0.22)
Total Alkalinity (mg/l)	30.25 (0.49)	30.31 (0.27)	32.38 (1.54)	32.87 (1.68)	34.02 (2.03)	35.48 (3.15)	36.60 (3.67)
Free carbon (iv) oxides (mg/l)	4.33 (0.16)	4.31 (0.18)	4.41 (0.22)	4.49 (0.31)	4.68 (0.22)	4.93 (0.43)	5.18 (0.48)

Standard deviation in parenthesis * $p<0.05$ across the row compared to the control

Table 2. Alkaline Phosphatase and Lactate Dehydrogenase activities in *Cyprinus carpio* Exposed to Sublethal Concentrations of *C. sativa* Crude leaf extract after 56 days

Parameters	**Tissue/Organs**	**Concentrations of *C. sativa* (mg/L)**						
		0.00 (Water)	**0.00 (Acetone)**	**1.88**	**3.75**	**7.50**	**15.00**	**30.00**
Alkaline Phosphatase (U/L)	Plasma	105.66 (3.45)	117.62 (2.51)	86.71 (3.91)	63.99 (5.29)*	59.99 (3.81)*	59.27 (4.29)*	51.631 (7.26)*
	Liver	175.07 (3.71)	163.62 (7.41)	152.99 (8.12)	128.53 (2.54)	120.72 (9.43)	85.81 (7.34)*	76.36 (4.53)*
	Gill	211.61 (1.69)	197.07 (8.56)	185.98 (8.05)	146.53 (1.67)	127.26 (2.28)*	125.26 (1.63)*	121.26 (3.44)*
Lactate Dehydrogenase (U/L)	Plasma	592.92 (5.21)	642.42 (6.43)	1210.05 (102.45)*	1250.39 (98.56)*	1730.03 (124.92)*	1922.09 (186.56)	2134.45 (321.76)*
	Liver	610.35 (34.61)	613.90 (75.09)	648.76 (30.18)	728.48 (112.09)	748.82 (96.28)	809.82 (106.48)*	884.38 (34.76)*
	Gill	609.06 (23.57)	669.08 (46.28)	665.55 (29.71)	649.41 (31.33)	866.61 (63.02)*	878.51 (39.48)*	933.12 (83.42)*

* $p<0.05$ across the row compared to the controls, standard deviation in parenthesis

Table 3. Activities of Serum Biochemical of *Cyprinus carpio* Exposed to Sublethal Concentrations of *C. sativa* Crude leaf extract after 56 days

Parameters	Concentrations of *C. sativa* (mg/L)						
	0.00 (Water)	0.00 (Acetone)	1.88	3.75	7.50	15.00	30.00
Aspartate aminotransferase (U/L)	293.67 (12.58)	294.00 (12.43)	295.17 (17.28)	320.83 (23.11)	322.67 (23.62)	326.80 (30.19)	385.33 (38.28)
Alanine aminotransferase (U/L)	13.35 (1.05)	16.67 (1.56)	25.67 (2.37)	28.00 (4.28)*	30.33 (4.61)*	34.00 (5.39)*	35.65 (6.82)*
Albumin (g/dl)	1.57 (0.01)	1.33 (0.02)	1.14 (0.01)	1.20 (0.01)	1.07 (0.02)	1.00 (0.03)	1.00 (0.06)
Bilirubin (mg/dl)	1.04 (0.00)	1.05 (0.00)	0.98 (0.00)	0.91 (0.00)	1.05 (0.01)	1.07 (0.01)	1.05 (0.01)
Total Bilirubin (mg/dl)	0.78 (0.00)	0.73 (0.00)	0.72 (0.00)	0.85 (0.00)	0.80 (0.00)	0.79 (0.01)	0.76 (0.01)
Uric acid (mg/dl)	71.11 (1.27)	76.40 (1.12)	71.08 (0.45)	88.33 (1.45)	114.92 (4.32)	130.54 (3.62)*	136.39 (4.72)*
Cholesterol (mg/dl)	432.87 (12.65)	385.47 (14.49)	113.95 (17.49)*	114.17 (21.69)*	213.80 (12.62)*	141.89 (12.56)*	112.52 (10.49)*

* $p<0.05$ across the row compared to the controls, standard deviation in parenthesis

Table 4. Total protein values of *Cyprinus carpio* Exposed to Sublethal Concentrations of *C. sativa* Crude leaf extract after 56 days

Parameters	Tissue/Organs	Concentrations of *C. sativa* (mg/L)						
		0.00 (Water)	0.00 (Acetone)	1.88	3.75	7.50	15.00	30.00
Total protein (g/dl)	Plasma	4.63 (1.23)	5.96 (0.89)	3.55 (2.48)	4.63 (2.07)	5.39 (0.94)	5.58 (1.08)	4.57 (1.09)
	Liver	3.30 (0.45)	3.30 (0.36)	3.68 (0.41)	3.87 (0.89)	5.58 (0.02)	5.58 (0.01)	5.58 (0.46)

Standard deviation in parenthesis

4. Discussion

The significant decrease in static renewable bioassay system pH as the concentrations of *C. sativa* increased, according to Adamu (2009), is attributed to the production of acidic metabolites by the plant leaf extract which Aleem (1987) suggested that the acidic condition of the water resulted to the decrease in the level of dissolved oxygen. However, Jalali Mottahari *et al*. (2013) reported that *C. carpio* are very tolerant to wide range of pH value thus the pH level recorded in this study may not be a threat to the test fish. The insignificant decrease in the level of dissolved oxygen may be attributed to the use of aeration during the study period. The temperature range recorded in this study were within the range for the fish as Mahdavi *et al*. (2013) reported 20 - 28°C for the culture of the test fish. Therefore, the temperature was within acceptable limits for fish culture (Swann, 2006). According to Capkin *et al*. (2006), total alkalinity above 20mg/L can significantly increase the survival rate of fishes thus the higher total alkalinity may be responsible for the 100% survival rate recorded in this study.

The value of total protein (3.55 (2.48) - 5.96 (0.89)) and albumin (1.00 (0.06) - 1.57 (0.01)) reported in the plasma of the test fish were within the range reported by Selamoglu *et al*. (2012) showing that the physiology of the normal fish is not affected by the exposure of the test fish to *C. sativa*. According to Adamu *et al*. (2013), the insignificant increase in liver total protein as the concentration of the plant extract increased may be due to a high demand of protein to metabolize the plant content or possibly due to haemo-concentration arising from fluid loss (Awasthy *et al.,* 2010). The increase liver total protein may also be attributed to the need to utilize protein as an energy source to compensate for increased energy demand to cope with leaf extract-induced stress (Dogan and Can, 2011). However, the decrease in plasma albumin recorded in this study may impede its function of transportation (Adamu and Kori-Siakpere, 2011) which may have resulted from the inhibitory effect of the plant on protein hydrolytic activity due to protease activity which corresponds to the decrease liver total protein level.

The hypocholesteremia condition recorded in the test fish exposed to plant leaf extract in this study is reported by Adamu and Kori-Siakpere (2011) in hybrid catfish exposed to tobacco leaf dust. Cholesterol level decreased as the concentration of the plant leaf extract increased which is accord with the findings of Samson *et al.* (2011). Alaa *et al*. (2010) asserted that cholesterol is the most important sterol occurring in plasma and red blood cells. If this assertion be true then, it is logical to add that the decrease in RBC content due to increased sublethal concentrations of *C. sativa* resulted in decrease cholesterol level in the blood of the exposed fish.

According to Martin *et al.* (1983), aminotransferase links carbohydrate and protein metabolism as it catalyzes their inter-conversion. The activities of these enzymes are directly proportional to the level of total protein and inversely proportional of cholesterol, an indication that the enzymes are catalyzing the inter-conversion of carbohydrate to protein in the liver. The activities of plasma aminotransferases were within the range reported by Selamoglu *et al*. (2012) in the test fish. It therefore noted that *C. sativa* caused increase in the activities of aminotransferases, as Amna (2011) reported increase in ALT and AST activities in rats exposed to *C. sativa*, which was concentration dependent. This therefore revealed that the plant has the potential of causing liver dysfunction. Alkaline phophatase and lactate dehydrogenase activities in the plasma of the test were within range for the test fish (Selamoglu *et al*., 2012). *C. sativa* has resulted in a significant decrease and an increase in these enzymes activities in the test during the period of study. According to Wright and Plummer (1974), ALP is employed to assess the integrity of plasma membrane and endoplasmic reticulum. Therefore, the significant decrease in ALP activity revealed that the plant extract has effect on plasma membrane of the test fish; showing a significant decrease in liver ALP activity. The decrease in liver ALP activity may be responsible for the decrease in protein levels in the test fish. As Pilo *et al.* (1972) reported that the decrease in ALP activity plays an important role in protein synthesis. In this investigation, the decrease in LDH activity indicated decrease metabolic activities of the exposed fish. The increase value of serum uric acid observed in this study could be due to the liver's ability to convert excess protein by way of deamination into less poisonous urea. This agrees with work of Martinez *et al.* (2004) who stated that fish under stress fish may mobilize protein to meet energy requirement needed to sustain increase in physiological activity.

In conclusion, the study revealed that the sublethal concentrations of crude leaf extracts of *Cannabis sativa* has effects on some biochemical parameters of common carp- *Cyprinus carpio* with the fish exposed to 30.00 mg/l showing more alterations in the determined biochemical parameters.

Acknowledgement

This research was supported by the study leave grant of the Research and Development office, Office of the Vice Chancellor, University of Jos, Jos, Nigeria.

References

Adamu KM. 2009. Sublethal effect of tobacco (*Nicotiana tobaccum*) leaf dust on enzymatic activities of *Heteroclarias* (a hybrid of *Heterobranchus bidorsalis* and *Clarias gariepinus*), *Jordan J Biol Sci.,* **2(4):** 151-158.

Adamu KM and Kori-Siakpere O. 2011. Effects of sublethal concentrations of tobacco (*Nicotiana tobaccum*) leaf dust on some biochemical parameters of Hybrid catfish (*Clarias gariepinus* and *Heterobranchus bidorsalis*). *Brazilian Arch Biol Technol.,* **54(1)**: 183-196.

Adamu KM, Isah MC, Baba TA and Idris TM. 2013. Selected liver and kidney biochemical profiles of hybrid catfish exposed to *Jatropha curcas* leaf. *Croatian J Fisheries,* **71**: 25-31.

Aleem SO. 1987. An assessment of tobacco waste for control of gastropod *Tympandomus fuscavis* (Linnaeus) in brackish water fish pond. *Aquaculture*, **73**: 19-25.

Akanji MA, Olagoke OA and Oloyede OB. 1993. Effects of chronic consumption of metabisulphate on the intergrity of the rat kidney cellular system. *Toxicol.,* **87**: 173-179.

Alaa G, Osman M, Ahmed S and Harabawy A. 2010. Hematotoxic and genotoxic potential of ultraviolet –A radiation on the African catfish *Clarias gariepinus* (Burchell, 1822). *J Fisheries Inter*., **5 (3):** 44-53.

Amna HM, Nabiela ME and Abdulrahim AG. 2011. The effect of *Cannabis sativa* on certain enzymes of clinical significance in rats and men. *J Pharmacognosis* ,**2(1):** 10-13.

APHA/AWWA/WPCF (American Public Health Association, American WaterWorks Association and Water Pollution Control Federation). 1985. Standard Methods of Examination of Water and WasteWater. 16th edition. APHA, Washington, D.C, U.S.A. 1268pp.

Audu BS, Adamu KM and Ufodike EBC. 2013. Behavioural response and opercular ventilation rate of Nile tilapia (*Oreochromis niloticus*) fingerlings after anesthesia with crude leaf extract of marijuana (*Cannabis sativa*), *Applied Sci Res J.,* **2(2):** 67-78.

Awasthy V, Vadiamudi VP, Koley KM, Awasthy BK and Singh PK. 2010. Biochemical changes after short term oral exposure to *Jatropha curcas* seeds in wistar rats. *Toxicol Inter.,* **17(2)**: 67-70.

Cao J, Chen J, Wang J, Wu X, Li Y and Yie L. 2013. Tissue distributions of fluoride and its toxicity in the gills of a freshwater teleost, (*Cyprinus carpio*). *Aquatic Toxicol.,* **130**: 68-76.

Capkin E, Altinok O and Karatan S. 2006. Water Quality and Fish size effect toxicity of Endosulfan, an organpchlorine pesticide to rainbow trout. *Chemosphere,* **64 (10)**: 1793-1800.

Dogan D and Can C. 2011. Hematological, biochemical and behavioural responses of *Oncorhychus mykiss* to dimethoate. *Fish Physiol Biochem.,* **37**: 951-958.

Emad HA, Khalid E and Mohamed AH. 2005. Toxicity of Cadmium and Copper and their effect on some biochemical parameters of *Mugil seheli. Egyptain J of Aquatic Res.,* **31(2):** 60-71.

Hampson AJ, Grimaldi M, Lolic M, Wink D, Rosenthal R and Axelrod J. 2000. Neuroprotective antioxidants from marijuana. Annals of New York Academy of Sci., **899**: 274-282.

Jalali Mottahari RS, Bozorgnia A, Ghiasi M, Farabi SMV and Toosi M. 2013. Impact of copper sulphate on haematological and some biochemical parameters of common carp (*Cyprinus carpio* L. 1758) in different pH. *World J Fish and Marine Sci.,* **5**: 486-491.

Koch JE. 2001. Delta 9-THC stimulates food intake in Lewis rats: effects of chow, high-fat and sweet high-fat diets. *Pharmacol Biochem Behaviour*, **68**: 539-543.

Mahdavi M, Hajimoradloo A and Ghorbani R. 2013. Effect of *Aloe vera* on growth parameters of common carp (*Cyprinus carpio*). *World J Medical Sci.,* **9**(1): 55-60.

Martin DW, Mayers PA and Rodwell VW. 1983. **Harper's Review of Biochemistry**. Lange Medical Publication, Maruzen Asia.

Martinez CBR, Nagae MY, Zaia CTBV and Zaia DAM. 2004. Acute morphological and physiological effects of lead in the neotropical fish *Prochilodus lineatus Brazilian J Biol.,* **64 (4)**: 797-807.

Mateeva A. 1995. Use of unfreindly plants against root knot nematodes. *Acta Horticulturae* **382**:178-182.

Mohamad RI, Ahmad RA and Milad K. 2011. Effects of sublethal concentrations of Chloramin T on the growth, survival, haematocirt and some blood biochemical parameters in common carp fry (*Cyprinus carpio*), *Aquaculture, Aquatic Conservation & Legislation Inter J Bioflux Society*, **4(3):** 280-291.

Pilo B, Asnani MV and Shah RV. 1972. Studies on wound healings and repairs in pigeon III. Histochemical studies on acid and alkaline phosphatase activity during the process. *J Animal Physiol.,* **19**: 205-212.

Rifat UK, Durrani FR, Chand N and Haseeb A. 2010. Influence of feed supplementation with *Cannabis sativa* on quality of broilers carcass. *Pakistan Veterinary J.,* **30(1):** 34-38.

Samson EA, King A, Esievo N, Shodeinde V and Shoyinka O. 2011. Evaluation of biochemical changes in *Clarias gariepinus* adults exposed to aqueous and ethanolic extracts of *Parkia biglobossa* pods. *African J Biotechnol.,* **10(2):** 234-240.

Sancho E, Ferrando MD and Andreau E. 1997. **A Textbook of Insect Toxicology**. Gorgan University Publication. pp 173-174.

Selamoglu TZ, Pinar DS, Faut GM, Orun I and Kakoolaki S. 2012. Effect of propolis on some blood parameters and enzymes in carp exposed to arsenic. *Iranian J Fisheries Sci*., **11(2):** 405-414.

Stratii Y I. 1976. Hemp and the Colorado beetle. *Zashchita Rasteniî*, **5**:61.

Swann L. 2006. A fish farmer's guide to understanding water quality. The Illinos-Indiana sea grant program Purdue University. http://aquanic.org/publicat/state/il-in/as-503.htm(accessed 20/11/ 2009).

Tokur B, Ozkutuk S, Atici E, Ozyurt G and Ozyurt CE. 2006. Chemical and sensory quality changes of fish fingers, made from mirror carp (*Cyprinus carpio* L. 1758), during frozen storage (-18ºC). *Food Chem.,* **99**: 335-341.

UNEP. 1989. United Nations Environment Programme. Estimation of the lethal toxicity of pollutants in marine fish and vertebrates, *Reference Methods for Pollution Studies* No.43, Ville-franche-sur-mer, France, Pp. 1-27.

Wright PJ and Plummer DT. 1974. The use of urinary enzyme measurement to detect renal damage caused by nephrotoxic compounds. *Biochem Pharmacol.,* **12**: 65.

JJBS

Volume 7, Number 2, June .2014
ISSN 1995-6673
Pages 153

Jordan Journal of Biological Sciences **Erratum**

An erratum of the following paper that was published in Volume 3, Number 3,(June) 2010 of Jordan Journal of Biological Sciences is given here by the authors.

The full citation of the paper is:

Shatnawi M, Al-Fauri A, Megdadi H, Al-Shatnawi M K, Shibli R, Abu-Rumman S and Al-Ghazwi A. 2010. *In vitro* Multiplication of *Chrysanthemum morifolium* Ramat and its Responses to NaCl Induce Salinity. *Jordan J. Biol. Sci*., **3**: 101-110.

Here are the errors that need to be taken into consideration:

1. Page 102- Materials and Methods Section, Subheading 2.4.1 *Effect of salinity on microshoots physiological responses*: This part should have been ended with the reference (Shatnawi *et al*., 2009). This is because this paper was based to a great extent on a previously reported data presented by the senior author during the Sixth Conference of the International of Society of Horticultural Science (ISHS). Most of the reported data and Tables were published as a preliminary paper in the Sixth Conference Proceedings on *In Vitro* Culture and Horticulture Breeding and should have been cited as (Shatnawi *et al*., 2009).
2. Page 103- Materials and Methods Section, Subheading 2.4.3 *Proline content*: This section should have been ended with the reference (Bates *et al*., 1973). This is because the method described in this section is based on this reference.
3. Accordingly, the following two references must be added to the References Section in their appropriate places:

-Bates LS, Waldren RP and Teare ID. 1973. Rapid determination of free proline for water stress studies. *Plant Sci*., **166**: 443-450.

-Shatnawi M, Fauri A, Shibli R, Al-Mazraawi M, Megdadi H and Makhadmeh I. 2009. Tissue culture and salt stress in *Chrysanthemum morifolium. Acta Hort. (ISHS).,* **829**: 189-196.

Here is a statement sent by the senior author to the Editor in Chief of the JJBS:

The authors apologize for missing to cite the original 2009 Conference Proceedings article in our 2010 full paper published in 2010 in JJBS. This was an unintentional honest error on our part. We had no intention of plagiarism or for any gain by doing so. This was a Conference Proceeding article and the ISHS Society does not claim copyrights infringement when an author publishes some of the preliminary data in a more complete form in a fully refereed journal. Indeed, ISHS stimulates such a process. The only requirement is that the reproduced material must be accompanied by a full citation. In this way, the 2010 paper published in JJBS should have given a full citation for our initial preliminary 2009 Conference Proceeding article published in Acta Hort. (ISHS) as given above. We sincerely apologize for any inconvenience that may result or may have resulted from this error in publication.

Any enquiries about this erratum notice should be referred to Dr. Mohamad Shatnawi at mshatnawi1@yahoo.com.au.

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