Hashemite Kingdom of Jordan

Jordan Journal of Biological Sciences

An International Peer-Reviewed Scientific Journal

Financed by the Scientific Research Support Fund

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

ISSN 1995-6673

Instructions to Authors

Scope

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

Type of Papers

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

Submission of Manuscript

Manuscript, or the essence of their content, must be previously unpublished and should not be under simultaneous consideration by another journal.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

Peer-review Process

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

Preparation of Manuscript

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

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

Title Page

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

Abstract

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

Keywords

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

Abbreviations

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

Introduction

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

Materials and Methods

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

Results

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

Discussion

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

Conclusions

This should briefly state the major findings of the study.

Acknowledgment

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

Tables and Figures

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

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

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

References

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

Reference to a journal publication:

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

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

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

Reference to a book:

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

Reference to a chapter in an edited book:

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

Conferences and Meetings:

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

Theses and Dissertations:

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

Nomenclature and Units

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

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

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

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

Math formulae

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

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

Copyright

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

Ethical Consent

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

Plagiarism

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

Galley Proofs

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

Publication Charges

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

Reprints

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

Disclaimer

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

Indexing

JJBS is indexed and abstracted by:

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

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

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

The Hashemite University

Deanship of Scientific Research and Graduate Studies

TRANSFER OF COPYRIGHT AGREEMENT

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

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

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

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

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

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

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

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

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

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

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

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

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

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

EDITORIAL PREFACE

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

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

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

In addition to being a hard-copy journal, JJBS is an open access journal which means that all contents are freely available the users and their institutions free of charge. Users are allowed to read, download, copy, distribute, print, search, or link to the full texts of the articles in this journal without asking for prior permission from the publisher or the author. This is in accordance with the BOAI definition of open access.

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

March, 2016

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

JJBS

Volume 9, Number3, September .2016
ISSN 1995-6673

Jordan Journal of Biological Sciences

CONTENTS

Original Articles

JJBS

Volume 9, Number 3,September .2016
ISSN 1995-6673
Pages 139 - 146

Jordan Journal of Biological Sciences

Analysis of Genotype × Environment Interaction for Grain Yield in Early and Late Sowing Date on Durum Wheat (*Triticum durum* Desf.) Genotypes

Leïla Haddad[1,*] , Hamenna Bouzerzour[2] , Amar Benmahammed[2] , Houcine Zerargui[3] , Abderrahmane Hannachi[4] , Adel Bachir[3] , Manel Salmi[1] , Abdelmalek Oulmi[2] , Zine El Abidine Fellahi[1] , Hind Nouar[2], Zahra Laala[1]

[1] *Agronomy Department, Faculty of Life and Natural Sciences, LVRBN Laboratory, Ferhat Abbas University, Setif 1, Algeria.*

[2] *Ecology and Biology Department, Faculty of Life and Natural Sciences, LVRBN Laboratory, Ferhat Abbas University, Setif 1, Algeria.*

[3] *Field Crop Institute Agricultural Research Station (ITGC), Setif, Algeria.*

[4] *Algerian National Institute of Agronomic Research (INRAA), Research Unit of Setif, Algeria.*

Received: May 16, 2016 Revised: June 17, 2016 Accepted: June 25, 2016

Abstract

Studying genotype × environment interactions and identifying the morphological traits contribution to the interaction are among the important tasks in crop breeding programs. The present investigation aims to analyze grain yield genotype × environment interaction and partitioning it into genotype × year and genotype × sowing date of 15 durum wheat (*Triticum durum* Desf.) genotypes. Trials were conducted during three consecutive cropping seasons, 2012/13, 2013/14 and 2014/15 and at two seeding dates (early vs late). The results indicate that genotype × year interaction was more significant than that of genotype x sowing date. Correlations of IPCA1 genotypic scores with several morphological plant traits indicated that 1000-kernel weight, number of spikes/m² and spikes weight/m² contributed significantly to grain yield interaction. Bousselam and Massara genotypes exhibited a general adaptation, with a grain yield mean above the overall average. Furthermore, the results suggested that the number of the testing years should be greater than the number of the seeding dates per year in studying genotype × environment interaction. Selection of stress tolerant genotypes should be based on 1000-kernel weight, number of spikes and spikes weight/m² to minimize grain yield interaction.

Keywords Triticum durum, G×E interaction, Sowing date, Grain yield, Regression, SSIndex.

1. Introduction

Wheat varieties react differently to a number of factors such as moisture stress, high temperature, weed infestation, soil fertility, disease pressure and sowing date, expressing a yield ranking change across environments, termed Genotype × Environment Interaction (GEI) (Bouzerzour and Refoufi, 1992; Basford and Cooper, 1998; Anwar et al., 2007, Subedi et al., 2007). Late planted crop, usually, suffers a yield decline due to the exposure to water deficit and high temperature at critical growth stages. These abiotic stresses hasten crop maturity, affect spikes number/m², pollen fertility, seeds set and seed weight (Chaudhry et al., 1995; Iqbal et al., 2001; Silva et al., 2014). Comparatively, optimum sowing date enhances the yield, as a result of relatively favorable weather conditions during the vegetative growth stage, and as such, this environment is relatively more suitable to discriminate between genotypes through their yielding abilities. Planting date management aimed generally to avoid or to minimize stress effects on crop performance (Tapley et al., 2013; Silva et al., 2014). GEI attenuates the relationship between phenotype and genotype, thereby reduces genetic progress.

Targeting high performing and stable genotypes is based on a better understanding of GEI pattern. Selection for specific or general adaptation is suggested and practiced as a way to increase genetic gain. Nouar et al. (2012) used AMMI model to study the GEI in durum wheat; they identified three sub-regions and concluded that selection for specific adaptation generated 10.5% genetic gain over selection for large adaptation. Furthermore, understanding the environmental and the genotypic

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

causes leading to genotype × environment interaction is an important issue in a plant breeding program while selecting parental material for crossing, and ideal test conditions.

Van Eeuwijk and Elgersma (1993) mentioned that regression of AMMI IPCA environmental scores against environmental covariates allows identifying environmental variables which contributed to the expression of the interaction. Van Oosterom et al. (1996) reported that the 10-day post flowering mean maximum temperature and changes in water satisfaction index during grain filling contributed to GEI in pearl millet. Similarly, genotype scores can be utilized to identify cultivar covariates which cause GEI. Annicchiarico and Iannucci (2008) correlated AMMI genotypic IPCA scores to mean values of various morphological traits to identify GEI causes. The objectives of the present study were to determine the magnitude of grain yield GEI and the traits contributing to the manifestation of the GEI under different sowing dates and to identify high-yielding and stable genotypes among 15 durum wheat genotypes..

2. Materials and Methods

2.1. Field Trials, Plant Material and Experimental Data

Field experiments were conducted during three successive growing seasons (2012/13 to 2014/15) at the Field Crop Station of the Agricultural Research Institute of Setif (ITGC-ARS, 05˚24′E, 36˚12′ N, 1081 masl), Algeria. A set of 15 durum wheat (Triticum durum Desf) genotypes (Table 1) was sown at two planting dates, early November and late December, in a complete randomized block design with three replications. Experimental plots had 6 rows of 5 m length and 0.2 m inter-row spacing. Plants were scored for Plant Height (PHT, cm), measured just before harvest, on three positions, taken at random along the diagonal of the elementary plot; Days to Heading (DHE), counted as the number of calendar days from January1st to the date when 50% of the spikes were half-way out of the flag leaf sheath. At maturity a 1 m long row-segment was harvested and used to measure the above ground biomass (BIO, g/m²), the number of spikes/m² (SN), and the Harvest Index (HI). Grain Yield (GY, g/m²) and a Thousand-Kernel Weight (TKW, g) were determined from the harvested plots. The Number of Kernels per Spike (NKS) was derived as the ratio of the number kernels/m² divided by the number of spikes/m². These traits were used as genotypic covariables.

Table1. Name and Pedigree of the genotypes tested during three successive years and two seeding dates at the ITGC-AES experimental site (Setif, Algeria).

Name	Pedigree
Bousselam	Heider/Martes//Huevos de Oro
Boutaleb	Hedba3/Ofanto
$Cyprus_2$	Gdo vz_{512}/Cit//Ruff/Fg/3/Ggo vz_{449}
Gta *durum*	Crane/4/Polonicum PI_{185309}//T.glutin enano/2* Tc60/3/Gll
Mansoura	Chinese spring/Mbb
Massara	Mrb_3/4/Bye*2/Tc/2/Zb/W/3/Cit
Massinissa	Ofanto/3/ Heider/Martes//Huevos de Oro's
Mbb	Local variety
Megress	Ofanto/Waha//Mbb
Moustakbel	Gta durum/Ofanto
$Setif_{2013}$	Unknown
Setifis	Heider/Martes//Huevos de Oro/3/Ofanto
Tajdid	Ofanto/3/ Heider/Martes//Huevos de Oro
Vitron	Turkey77/3/Jori/ Anhinga//Flamingo
Waha	Plc/Ruff//Gta's/3/Rolette

2.2. Data Analysis

Grain yield data were subjected to one factor analysis of variance per seeding date, to test genotype effect; then subjected to a combined analysis of variance over seeding dates to test seeding date, genotype and genotype x seeding date effects and combined over seeding dates and years to test year, seeding date, genotype main effects and their interactions. The genotype × year interactions within seeding date and over all environments, taking the combination year × seeding date as environment, were analyzed by the AMMI model. In order to achieve a better understanding of genotype × environment interaction, values of the pheno-morphological measured traits served as covariates and were correlated to the AMMI IPCA1 genotypic scores (Van Eeuwijk and Elgersma, 1993). Significant correlations suggested that any separation of the genotypes on the AMMI1 biplot was attributed to the relevant genotypic covariate, highlighting the importance of that covariate to the G × E interactions and suggesting biological interpretations of the factors causing G × E interactions. All statistical analyses were performed using Cropstat software (Cropstat, 2007).

3. Results and Discussion

3.1. Yield variability between years and sowing dates

Grain yield means of early seeding were 511.0, 93.7, and 227.5 g/m² in 2013, 2014, and 2015, respectively. Those of late seeding were 469.9, 62.0, and 158.1 g/m², respectively. Relative yield reduction, measured under late planting, was 8.0, 33.8, and 30.5% of early sown grain yield, in 2013, 2014, and 2015, respectively. These results suggested the potential of optimum planting date and corroborated results of several research studies (Bassu et al., 2009; Tapley et al., 2013; Silva et al., 2014). Correlation coefficients between years for early (rGY13/GY14= -0.213ns, rGY13/GY15= -0.049ns, rGY14/GY15= -0.024ns) and lately planted trials were insignificant (rGY13/GY14= -0.057ns, rGY13/GY15= 0.122ns, rGY14/GY15= 0.460ns). The correlations between sowing dates within year were insignificant for two seasons 2013 and 2015 (rGYS113/GYS213 = 0.270ns, rGYS115/GYS215 = -0.057ns), and significant for the 2014 trial (rGYS114/GYS214 = 0.604*). These results suggested the significant effect of both genotype × year and genotype × seeding date interactions.

The combined analysis of variance indicated that the differences among environments (combination of year × sowing date) explained 86.0% of the total grain yield variation. The partitioning of these differences, among years and among sowing dates, explained 84.3 and 1.6%, respectively (Table 2). Differences among genotypes explained 2.4%, while genotype x environment interaction (G×E) explained 11.6% of grain yield treatment sum square. The G×E portioning indicated that genotype x year (G×Y), genotype x sowing (G×S) and genotype x sowing date × year (G×S×Y) components accounted for 53.1, 18.3, and 28.6%, respectively. Year main effect, G×Y and G×S×Y interactions accounted for a large proportion of treatment sum square and total G×E variance, respectively (Table 2). The assessment of the relative importance of these sources of variations is justified to take advantage of the GEI. Zhang et al. (2006) reported that contributions of the location, year, and sowing dates are proportionally greater than the main effect of the genotype and interactions. AMMI analysis of variance of grain yield of the three years per sowing date indicated that early sowing was more discriminating between genotypes than late seeding environment. In fact genotype sum square represented 5.27 and 3.81% of the treatment sum square (TSS) for early and late seeding, respectively. G×Y sum square was slightly higher under early than under late sowing (Table 3), suggesting more reactive conditions under early sowing.

High proportion of the G×Y interaction sum square (G×Y SS) was explained by the first AMMI Interaction Principal Component (IPCA1), under both growing conditions of early (77.06%) and late sowing (95.54%). This indicated that biplot AMMI1 sufficiently described the behavior of the tested genotypes (Table 3). Biplot of grain yield measured under early sowing indicated that years were clearly separated with 2014 being the less favorable (93.7 g/m²) and 2013 the most favorable (511.0 g/m²) to grain yield expression (Figure 1).

Table 2. Combined analysis of grain yield for 15 genotypes tested during three successive years and two seeding dates at the ITGC-AES experimental site (Setif, Algeria).

Source	Df	SS	MS	%SS
Treatment	89	9681034.8	108775.7**	100.0
Environment (E)	5	8328550.0	1665710.0**	86.0
Year (Y)	2	8159450.0	4079720.0**	84.3
Sowing (S)	1	151815.0	151815.0**	1.6
Y × S	2	17294.0	8647.0**	0.2
Repetition / E	12	2113.9	176.1^{ns}	
Genotype (G)	14	229352.0	16382.3**	2.4
G × E	70	1123110.0	16044.5**	11.6
G × Y	28	596493.0	21303.3**	53.1
G × S	14	205495.0	14678.2**	18.3
G×Y×S	28	321125.0	11468.7**	28.6
Residual	168	33675.6	193.5	

Table 3. AMMI analysis of variance for grain yield of 15 genotypes tested during three successive years and two seeding dates at the ITGC-AES experimental site (Setif, Algeria).

		Early sowing			Late sowing		
Source	Df	SS	MS	%SS	SS	MS	%SS
Treatment	44	4920520.00	111830.00**	100.00	4608690.00	104743.00**	100.00
Year (Y)	2	4085010.00	2042505.00**	83.02	4091730.00	2045865.00**	88.78
Rep / Y	6	1470.08	245.01^{ns}	--	1825.02	304.17^{ns}	--
Genotype (G)	14	259410.60	18529.33^{ns}	5.27	175436.40	12531.17^{ns}	3.81
G × Y	28	576111.00	20575.39**	11.71	341508.00	12196.71**	7.41
$IPCA_1$	15	443973.00	29598.20**	77.06	326289.00	21752.60**	95.54
Deviation	13	132136.80	10164.37**	22.94	15218.61	1170.66^{ns}	4.46
Residual	83	33098.40	398.80		69878.70	841.91	

Year 2013 (IPCA1 score of -15.62), and 2015 (IPCA1 score of 10.87) were the most interactive environments, classifying differently the tested genotypes. Cyprus2 and MBB were low yielding and Setifis and Setif2013 were high performing, in 2013; while in 2015, Mansoura and Moustakbel were low yielding and Waha and Cyprus2 were high yielding varieties (data not shown). Based on the genotype main effect, Mansoura showed a low yielding ability while Setifis and Setif2013 expressed a high yielding capacity under early sowing (Figure 1). Genotypes, with a grain yield mean above average, were Waha, Bousselam, Boutaleb, Massara, Setif2013, and Setifis. Having a below-average grain yield mean, Tajdid, Vitron, Gaviota and Massinissa expressed a relatively high stability, their IPCA1 score varied from -1.29 to 0.74. The most unstable entries were Bousselam, Setifis, with an IPCA1 score below -5.00, and Waha and Cyprus2, with an IPCA1 score above 7.00 (Figure 1).

Biplot of late planting indicated that years were clearly separated with 2015 being the less favorable (62.1 g/m²) and 2013 the most favorable (469.9 g/m²) to grain yield expression (Figure 2).

2013 with an IPCA1 score of 14.79, and 2014, with an IPCA1 score of -8.29 were the most interactive environments.

In 2013, Tajdid and Waha were the lowest yielding entries while Mansoura and Bousselam were the best yielding varieties. In 2014, Bousselam and Mansoura were the least performing, while Megress and Massara were top yielding (data not shown). Based on the genotype main effect, Tajdid, Waha, Cyprus2 and MBB exhibited low yielding ability.

Vitron, Bousselam and Megress expressed a high yielding capacity (Figure 2). Entries with a grain yield mean above average were Vitron, Bousselam, Megress, Moustakbel, Massara, and Setifis. Only Bousselam, Setifis and Massara expressed above average grain yield under both growth conditions of early and late seeding (Figures 1, 2). Boutaleb, MBB and Gaviota were relatively more stable, with an IPCA1 score varying from -1.17 to 1.31. Among these three entries Gaviota had above average grain yield. Unstable entries were Tajdid, Waha and Cyprus2, with an IPCA1 score below -4.00, and Bousselam and Mansoura with an IPCA1 score above 7.00 (Figure 2). As the focus of the present study was genotype × year interaction within seeding date treatments, the biplot analysis confirmed that grain yield was more affected by genotype × year than by genotype × seeding date interactions. Several previous studies also showed that differences among consecutive years are larger than differences among locations and among seeding dates within a region (Coventry et al., 2011; Benin et al., 2014). The genotype × year interaction was greater in the early seeded treatment than in late seeded treatment. Year effect on grain yield variation in both sowing dates was the largest. These results suggested that increased number of testing years is justified more than the number of seeding dates per year. Studying the effects of years, locations and sowing dates on the performance of wheat genotypes, Benin et al. (2014) reported that years and locations contributed more to G×E interaction, explaining 24.3% and 12.5%, respectively; while seeding date contributed less, explaining 7.0% only.

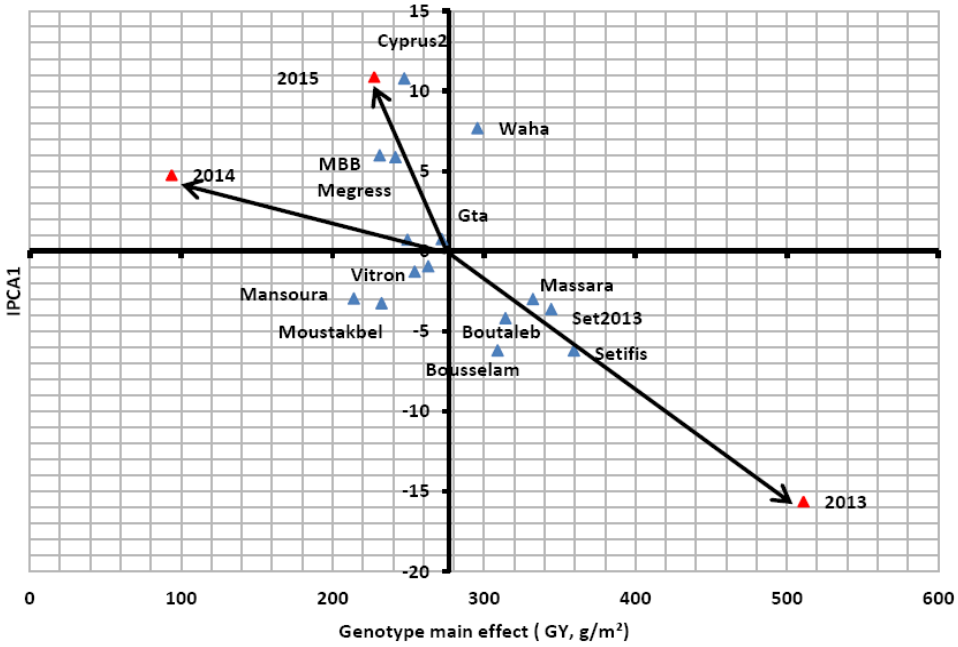


Figure 1. AMMI$_1$ biplot of grain yield for 15 durum wheat genotypes grown under early planting at the ITGC-AES experimental site (Setif, Algeria).

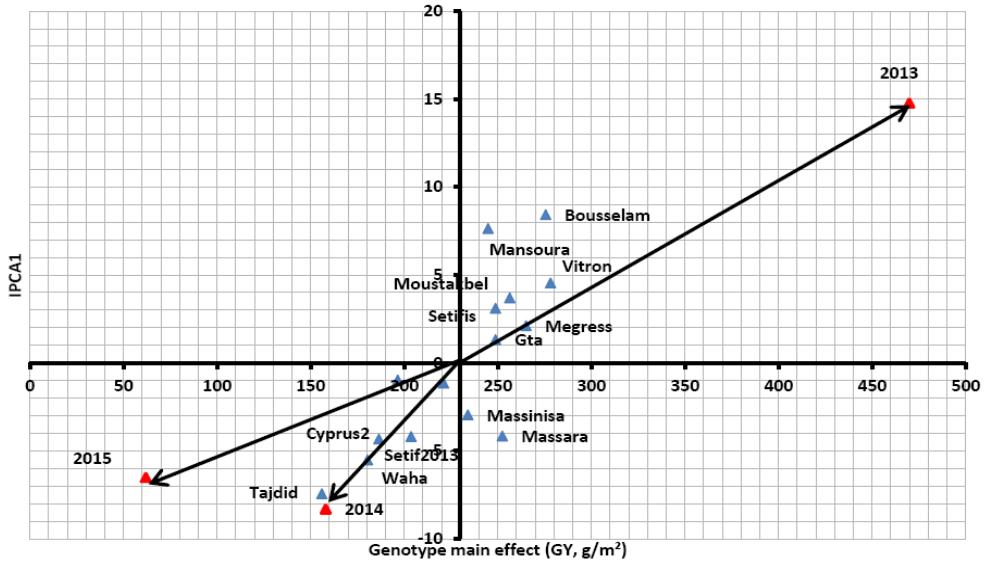


Figure 2. AMMI$_1$ biplot of grain yield for 15 durum wheat genotypes grown under late planting at the ITGC-AES experimental site (Setif, Algeria).

Taking year × seeding date combination as environment, AMMI analysis indicated that G×E interaction is modeled on more than one dimension; with the first three IPCA significant, contributing 56.5%, 35.6% and 5.9 %, respectively (Table 4).

Table 4. AMMI analysis of variance for grain yield of 15 durum wheat genotypes tested in six environments.

Source of variation	df	SS	MS	%SS
Treatment	89	9681034.8	108775.7**	100
Environment (E)	5	8328570.0	1665714.0**	86
Rep/E	12	2113.9	176.2	--
Genotype (G)	14	229351.8	16382.3ns	2.4
G × E	70	1123113.0	16044.5**	11.6
IPCA$_1$	18	634371.0	35242.8**	56.5
IPCA$_2$	16	399735.0	24983.4**	35.6
IPCA$_3$	14	66135.3	4724.0*	5.9
Deviation	22	22872.8	1039.6*	--
Pooled residual	168	34730.3	206.7	--
Total	269	9716090.0		

AMMI2, which explained 92.1% of the sum square of the interaction, indicated that the tested environments classified differently the evaluated genotypes (Figure 3).

Environment E5 was opposed to environment E2, and environment E1 was opposed to E4, E3 and E6 which formed a homogeneous group of environments, classifying similarly the tested genotypes. Based on their high scores, on the IPCA1 and IPCA2, E5, E1 and E2 were the most reactive environments generating interaction. Environment E6 was the least discriminating as indicated by its low score value, also, genotype classification in this environment is similar to the average classification over all environments. Gta durum and Massinissa were the most stable due to their position near the origin (Figure 3). Massara, Setifis, Sétif2013, Bousselam and Boutaleb were best expressed in E1; Waha, Cyprus2 and Tajdid in E5; MBB and Megress in E4 and E3; while Vitron, Mansoura and Moustakbel had high grain yield in E2 (Figure 3). Based on the AMMI2 biplot analysis, most of the tested genotypes expressed specific adaptation. Nominal yield, which helps to apprehend the general adaptability of each cultivar and to identify genotypes that yielded best at specific location IPCA1, classified the tested environment into two recommendation domains. The first recommendation domain is constituted by five environments (E1, E2, E3, E4 and E6) where Setifis was the leading genotype. Environment E5 constituted a separate recommendation domain where cultivar Waha was the winner (Figure 4).

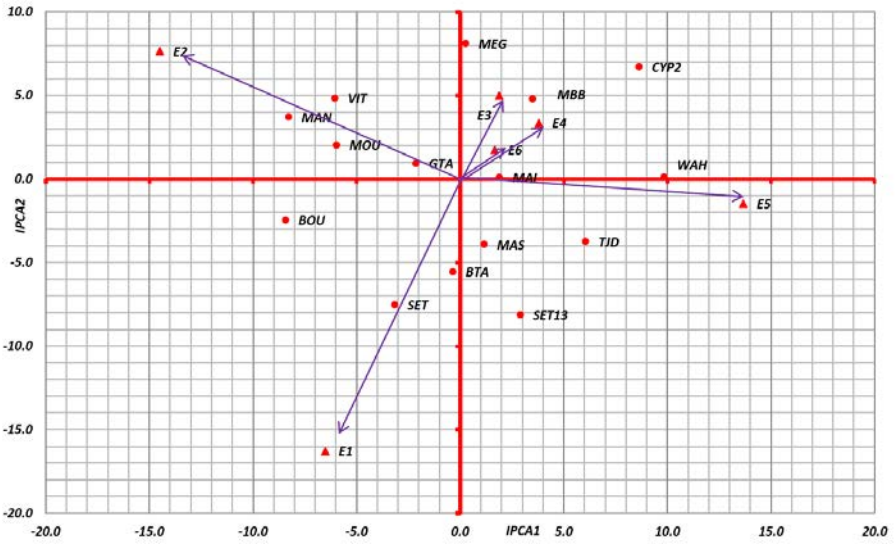


Figure 3. AMMI$_2$-biplot of grain yield for 15 durum wheat evaluated in six environments (Mas= Massara, Set13= Sétif2013 ,Cyp2= Cyprus2, MBB= Mohammed Ben Bachir, WAH= Waha, GTA= Gaviota durum, VIT= Vitron, MEG = Megress, SET= Setifis, BOU= Bousselam, MAN= Mansoura, MAI= Massinissa, TJD= Tajdid, MOU= Moustakbel, BTA= Boutaleb, E1 = early seeding 2013, E2 = late seeding 2013, E3= early seeding 2014, E4 = late seeding 2015, E5= early seeding 2015, E6 = late seeding 2015).

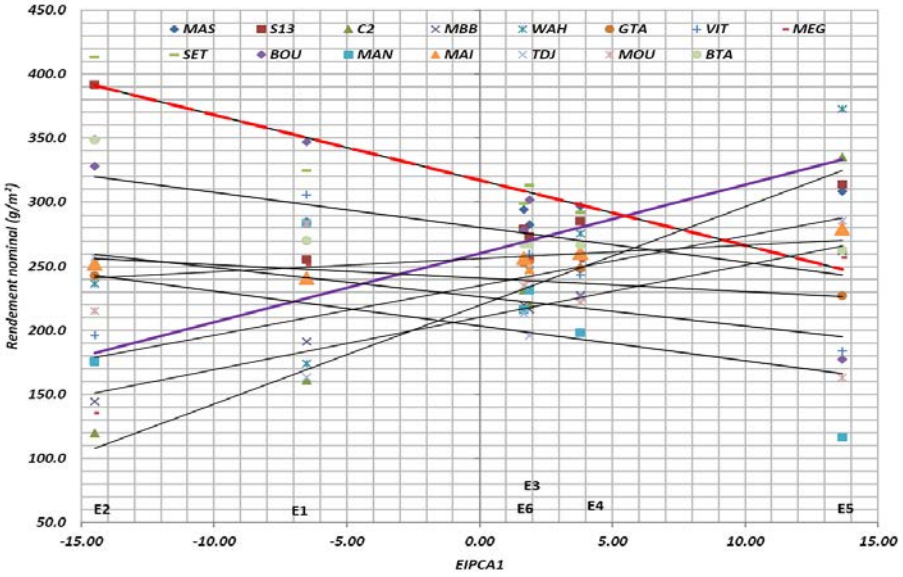


Figure 4. Nominal grain yield variation for 15 durum wheat genotypes evaluated in six environments (Mas= Massara, Set13= Sétif2013 ,Cyp2= Cyprus2, MBB= Mohammed Ben Bachir, WAH= Waha, GTA= Gaviota durum, VIT= Vitron, MEG = Megress, SET= Setifis, BOU= Bousselam, MAN= Mansoura, MAI= Massinissa, TJD= Tajdid, MOU= Moustakbel, BTA= Boutaleb, E1 = early seeding 2013, E2 = late seeding 2013, E3= early seeding 2014, E4 = late seeding 2015, E5= early seeding 2015, E6 = late seeding 2015).

3.2. Traits Implicated in GEI Expression

As genotypic IPCA1 scores summarize a sizeable part of the GEI expressed, any morphological traits significantly correlated with the PC1 scores are causative of GEI. IPCA1 showed negative correlations with 1000-kernelweight ($P < 0.01$) and plant height ($P < 0.05$) indicating that lower height and lower grain weight tended to contribute to GE interaction. Significant correlations existed between IPCA1 scores and thousand-kernel weight (rIPCA1/TKW = -0.452*), between IPCA1 and number of spikes/m² (rIPCA1/SN = -0.500*) and between IPCA1 scores and spikes weight/m² (rIPCA1/SW = -0.479*). These correlations indicated that variation in the expression of these plant traits led some genotypes to achieve a high grain yield in some environments but less so in others. These traits explained 21%, 25% and 23% of grain yield GEI, respectively. These results corroborated findings of Nachit et al. (1992) who mentioned that plant height and fertile tillers explained up to 59% of the manifestation of grain yield GEI in rainfed durum wheat. Mohammadi and

Amri (2013) noted that plant height contributed most to GEI in rainfed durum wheat yield. Van Ginkel et al. (1998) showed the importance of number of spikes/m² in situations in which wheat plants experienced late-season drought stress during grain filling, as this is the case under delayed sowing. Van Eeuwijk et al. (1996) reported that differences in diseases resistance are implicated in the expression GEI in wheat. Yan and Hunt (2001) mentioned that differences in plant height and cycle duration are contributors to grain yield GEI. Consequently, thousand kernel weight, spike number and spike weight could be used as the basis for selecting high yielding genotypes less sensitive to variable environments.

4. Conclusion

The results of the present study show the potential of the optimum planting of durum wheat under semi-arid conditions and the presence of both genotype × year and genotype × seeding date interactions. The combination of year x sowing date, with 86.0%, was the most important source of grain yield variation. Differences among genotypes explained 2.4%, and genotype × environment interaction (G×E) explained 11.6%. Early sowing was more discriminating between genotypes than late seeding environment. High proportion of the G×Y interaction sum square was explained by the IPCA1, under early (77.06%) and late sowing (95.54%) conditions. In general, genotypes with a grain yield mean above average were less stable than those with a grain yield mean below yield average, under early and late sowings. Bousselam and Massara genotypes expressed above average grain yield under both growth conditions of early and late plantings. As year effect on grain yield variation in both sowing dates was the largest, the results suggested that increased number of testing years is justified more than the number of seeding dates per year. IPCA1 scores were negatively correlated with 1000-kernel weight, number of spikes/m² and spikes weight/m², suggesting that variation in the expression of these plant traits led some genotypes to achieve a high grain yield in some environments and low in others. Consequently, thousand kernel weight, spike number and spike weight could be used as the basis for selecting high yielding genotypes less sensitive to environmental variations.

Acknowledgement

This study was undertaken as a part of the project CNEPRU N° F01220140023 titled 'Variability, genetic determinism and selection of cereals under semi-arid conditions of the Algerian eastern high plains', financed by the Algerian Ministry of Higher Education and Scientific Research. Special thanks are extended to the personnel of the ITGC-ARS and INRAA-RUS, particularly to Mrs Djamel Sersoub, Houcine Zerargui, Abderrahmane Hannachi, Adel Bachir and Zine El Abidine Fellahi for providing seeds, helping in the management of the trials and data collection.

References

Annicchiarico P and Iannucci A. 2008. Breeding strategy for *Faba bean* in Southern Europe based on cultivar responses across climatically contrasted environments. Crop Sci, **48**: 983–991.

Anwar J, Khan SB, Rasul I, Zulkiffal M and Hussain M. 2007. Effect of sowing dates on yield and yield components in wheat using stability analysis. IJAB, **9:** 129–132.

Basford KE and Cooper M. 1998. Genotype x environment interactions and some considerations of their implications for wheat breeding in Australia. Aust. J. Agric. Res, **49**: 153–174.

Bassu S, Asseng S, Motzo R and Giunta F. 2009. Optimizing sowing date of durum wheat in a variable Mediterranean environment. FIELD CROP RES, **111**: 109–118.

Benin G, Storck L, Marchioro VS, Todeschini MH, Milioli AS and Woyann LG. 2014. Effects of years, locations and sowing date on the spring wheat yield performance.1Rev. Fac. Agron, **113**: 165–173.

Bouzerzour H and Refoufi B. 1992. Effect of sowing date and rate, and site environment on the performance of barley cultivars grown in the Algerian high plateaus. Rachis, **11**: 19–24.

Chaudhry MH, Anwar J, Hussain F and Khan FA. 1995. Effect of planting time on grain yield in wheat varieties. J. Agric. Res, **33**: 103–108.

Coventry DR, Gupta DK, Yadav A, Poswal RS, Chhokar RS, Sharma RK, Yadav VK, Gill SC, Kumar A, Mehta A, Kleemann SG, Bonamano A and Cummins JS. 2011. Wheat quality and productivity as affected by varieties and sowing time in Haryana, India. FIELD CROP RES, **123**: 214–225.

Cropstat 7.2. 2007. Free Software package for windows. International Rice Research Institute. IRRI. Manila.

Iqbal MS, Yar A, Ali A, Ansern MR, Iqbal J and Akram MH. 2001. Effect of sowing dates and seed rate on grain yield of wheat (CV.93-BT-022). J. Agric. Res, **39**: 217–21

Mohammadi R and Amri A. 2013. Genotype × environment interaction and genetic improvement for yield and yield stability of rainfed durum wheat in Iran. Euphytica, **192**: 227–249.

Nachit MM, Nachit G, Ketata H, Gauch HG and Zobel RW. 1992. Use of AMMI and linear regression models to analyse genotype-environment interaction in durum wheat. Theor. Appl. Genet, **83**: 597–601.

Nouar H, Bouzerzour H, Haddad L, Menad A, Hazmoune T and Zerargui H. 2012.Genotype × environment interaction assessment in durum wheat (Triticum durum Desf) using AMMI and GGE models. Advances Environmental Biology, **6**: 3007–3015.

Silva RR, Benin G, Marchese JA, Silva ED and Marchioro VS. 2014. The use of photothermal quotient and frost risk to identify suitable sowing dates for wheat. Acta Scientiarum. AGRON J, **36**: 99–110.

Subedi KD, Ma B.L and Xue AG. 2007. Planting date and nitrogen effects on grain yield and protein content of spring wheat. Crop Science, **47**: 36–44.

Tapley M, Ortiz BV, Santena EV, Balkcomb KS, Maska P and Weavera DB. 2013. Location, Seeding Date, and Variety Interactions on Winter Wheat Yield in Southeastern United States. AGRON J, **105**: 509–518.

Van Eeuwijk FA and Elgersma A. 1993. Incorporating environmental information in an analysis of G × E interaction for seed yield in perennial ryegrass. Heredity, **70**: 447–457.

Van Eeuwijk FA, Denis JS and Kang MS. 1996. Incorporating additional information on genotypes and environments in models for two-way genotype by environment tables, *in:* Kang MS and Gauch HG. Genotype-by-Environment Interaction, (Eds.),CRC Press, Boca Raton, FL.

Van Ginkel M, Calhoun DS, Gebeyehu S, Miranda A, Tian-you C, Pargas Lara R, Trethowan RM, Sayre K, Crossa J and Rajaram S. 1998. Plant traits related to yield of wheat in early, late, or continuous drought conditions. Euphytica, **100**: 109–121.

Van Oosterom EJ, Bidinger FR, Mahalakshimi V and Rao KP. 1996. Effect of water availability pattern on yield of pearl millet in semi-arid tropical environments. Euphytica, **79**: 165–173.

Yan W and Hunt LA. 2001. Interpretation of genotype x environment interaction for winter wheat yield in Ontario. Crop Sci, **41**: 19–25.

Zhang Y, He Z, Zhang A, Van Ginkel M and Ye G. 2006. Pattern analysis on grain yield performance of Chinese and CIMMYT spring wheat cultivars sown in China and CIMMYT. Euphytica, **147**: 409–420.

JJBS

Volume 9, Number 3 September .2016
ISSN 1995-6673
Pages 147 - 154

Jordan Journal of Biological Sciences

Experimenting Two Cryopreservation Techniques (Vitrification and Encapsulation-Dehydration) as Approaches for Long- term Conservation of in vitro Grown Shoot Tips of Wild Fennel

Rida A. Shibli[1,*], Fayha Al Hawmdeh[1], Mahmud Duwayri[1], Nazir Hadidi[1], Tamara S. Al-Qudah[2] , Reham W. Tahtamouni [3], Laila S. Younes [4]and Ahmad Zateemeh[5]

[1] *Department of Horticulture and Agronomy, Faculty of Agriculture, University of Jordan, Amman, Jordan*

[2] *Hamdi Mango Center for Scientific Research (HMCSR), University of Jordan, Amman, Jordan*

[3] *Department of Applied Sciences, Princess Alia University College, Al- Balqa Applied University, Amman, Jordan*

[4]*Agricultural and Animal Research Center,Tripoli, Libya*

[5] *Department of Applied Sciences, Ajloun University College, Al- Balqa Applied University, Ajloun, Jordan.*

. Received: March 24, 2016 Revised: June 20, 2016 Accepted: June 28, 2016

Abstract

Wild fennel (*Foeniculum vulgare* Mill.) is a medicinal plant that is native to Jordan as well as to most of Mediterranean region. Wild fennel is considered an aromatic medicinal plant and is used in cooking as a spice, and in folk medicine for the treatment of gastrointestinal and respiratory tract diseases. Due to the high international demand for this plant, the extensive uncontrolled collection of wild fennel became highly threatened species. In the present study, two cryopreservation techniques (vitrification and encapsulation-dehydration) were experimented as approaches for long term conservation of in vitro grown shoot tips of wild fennel. The results obtained from vitrification experimental part showed that survival and regrowth rates varied with type of loading solution, and the maximum survival (80%) and regrowth (65 and 60%) rates were recorded in shoot tips (STs) preloaded with either (2M glycerol + 0.4M sucrose) or (5% Dimethylsulfoxide, DMSO + 0.5 M sucrose), respectively. Also, data revealed that survival and regrowth rates of wild fennel STs were determined by the type of plant vitrification solution, as the highest survival and regrowth rates (85 and 60%, respectively) were obtained in STs cryoprotected with plant vitrification solution type 2 (PVS2) before exposure to Liquid Nitrogen (LN). Moreover, the best regrowth rate (65%) was recorded in encapsulated STs of wild fennel with 21.1% moisture content obtained after chemical dehydration in MS liquid media supplemented with 0.75 M sucrose for 1 day followed by air dehydration for 6 hrs. However, more research is still needed to optimize the pretreatments protocols prior exposure to LN in terms of ingredients, concentration and duration to improve regrowth rates.

Keywords: Cryopreservation, Encapsulation- dehydration, Vitrification, Wild fennel.

1. Introduction

For decades, people have used the vegetation around them for food, fuel and medicinal purposes. Consequently, the extensive use of plant resources resulted in environmental degradation. So, many strict regulations were developed to conserve plant resources, such as preventing cutting down indigenous trees and over grazing in addition to strict enforcement of existing rules/regulations of nature reserves (Ghazanfar et al., 2015). But, despite these regulations, plants are still a way from being safeguarded.

Wild fennel (Foeniculum vulgare Mill.) is a medicinal plant that belongs to Apiaceae family (He and Huang, 2011). This plant is native to Southern Europe and Mediterranean region including Jordan. Through history, wild fennel was considered a well-known aromatic medicinal plant, as all parts of it including shoots, leaves and fruits were used in cooking as a spice, and in folk medicine for the treatment of gastrointestinal and respiratory tract ailments (Raffo et al., 2011). Also, wild fennel is considered an important economic crop, and is

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

traded internationally to be utilized in phytoindustry (Raffo et al., 2011). However, the medicinal potential of wild fennel is due to the presence of many distinguished compounds, such as essential oil, fatty acid, phenylpropanoids, monoterpenids, sesquiterpenes and saponins (He and Huang, 2011) in most plant parts (shoots, roots, leaves, and seeds). Additionally, some elite compounds that were isolated from wild fennel, such as, trans-anethole, estragole, fenchone, sesquiterpenoids, coumarins and polyphenolics, were found to possess antimicrobial activities (Gulfraz et al., 2008). Meanwhile, in Jordan, wild fennel is suffering from serious degradation threats, as it is the case of many wild medicinal plants, due to the extensive uncontrolled collection by the locals for food and medicinal purposes (Royal Botanic Garden (RBG), 2015). This makes conservation of this plant of a high priority.

The ultimate task of plant conservation strategies is how is to ensure a sustainable supply of plant resources without altering the variety of genes or destroying the natural habitats and ecosystems (Kasagana and Karumuri, 2011). To fulfill this task, biotechnology protocols have been practiced to conserve plant genetic resources from loss and decline. These protocols include, slow growth conservation and cryopreservation. In slow growth conservation, inhibited or minimal growth is achieved by exposing the stored plant material to retardants, such as ABA, low environmental storage conditions (pressure, oxygen, temperature or light intensity), minimal growth media and the addition of osmotic agents, such as sucrose, mannitol or sorbitol to the culturing media (Shibli et al., 2006; Rabba'a et al., 2012; Sharaf et al., 2012; Tahtamouni et al., 2015). This can keep the plant material preserved for 1-15 years without the need for frequent subculturing (Rao, 2004). Cryopreservation is another storage technique that involves storage of plant material of a wide range of plant genotypes at ultra-low temperatures in liquid nitrogen (LN; -196°C) where cell division, metabolic and biochemical activities remain stopped, and yet plant material can be stored for unlimited time without alteration and deterioration (Engelmann, 2004; Shibli et al., 2006; Tahtamouni et al., 2015). For vegetatively propagated species, the best organs for cryopreservation are shoot apices or meristems, as they are virus-free, true to type, genetically stable and usually record high and recovery rates after cryopreservation (Micula et al., 2011).

In the last three decades, different protocols for cryopreservation were developed to meet the cryogenic needs of most plants species. These protocols include vitrification, encapsulation-dehydration, encapsulation-vitrification, and droplet-vitrification (Engelmann, 2004). Vitrification protocol involves supercooling of plant materials at ultra-low temperature after exposing them to a highly concentrated cryoprotectant, and yet the cryoprotectant inside the plant cell will solidify into a glass state without formation of ice crystals after exposure to liquid nitrogen (Sakai and Englemann, 2007). Encapsulation-dehydration is another cryopreservation protocol, in which shoot tips, somatic embryos or callus cells are encapsulated within alginate beads and cultured in a medium containing elevated concentrations of an osmotic agent (Fabre and Dereuddre, 1990). Encapsulation-dehydration is widely practiced on plants, because it is easily handled and maintains high rates of recovery after cryopreservation, this is due to the beads surrounding the explant which reduces the shock of exposure to liquid nitrogen (Shibli et al., 2006; Shatnawi et al., 2011).

The present study aims to experiment two cryopreservation techniques (vitrification and encapsulation- vitrification) as approaches for long term conservation of in vitro grown wild fennel shoot tips to ensure a sustainable true to type supply of this important plant for research and industry. ..

2. Materials and Methods

2.1. Mother Stock Establishment of Wild Fennel

Sterilized seeds of wild fennel were inoculated on a full strength (4.4.g/L) hormone free MS (Murashige and Skoog, 1962) solid media supplemented with (30 g/L) sucrose. Then, cultures were kept in the growth room under daily regime of 16/8hr light/ dark regime (photosynthetic photon flux density (PPFD) = 40-45 µmole/m-2/s-1, light and 8-h dark photoperiod and 24 ± 1 °C till seed germination. After seed germination, the resulted microplants were transferred to a fresh hormone free MS media, prepared as described above for one month and kept under the same growth room conditions described earlier. Next, a preliminary experiment was conducted to decide the type and constituents of a shoot multiplication media that would yield the best multiplication rate by subculturing wild fennel microshoots into MS media, prepared as described earlier and supplemented with various types and levels of cytokinins plus 0.01 mg/L NAA and kept under the same growth conditions, as described before, and the best multiplication rate was obtained in MS media supplemented with 1.5 mg/L BA and 0.01 mg/L NAA (data not shown). Subculturing was performed every 4 weeks by subdividing the microshoots aseptically until a massive mother stock was obtained.

2.2. Cryopreservation

All the conducted cryopreservation experiments have followed the protocol reported by Rabba et al. (2012).

2.2.1. Cryopreservation Using Vitrification Technique

Effect of Loading Solution

Shoot Tips (STs) were subculutred into a preculture media consisted of MS hormone free media plus (0.3 M) sucrose and kept under complete

dark conditions for three days. Then the STs were transferred into cryovials and loaded with 1 ml of different cocktails of loading solutions composed of Hormone Free (HF)-liquid media supplemented with either of (1 M sucrose, 0.4 M sucrose + 2 M glycerol, or combinations of sucrose [0.25, 0.5 M] + dimethylsulfoxide (DMSO) [5 or 10%] at 25 ^{0}C for 20 min before being exposed to plant vitrification solution 2 (PVS2) which consisted of 30% glycerol, 15% ethyleglycol (EG), 15% DMSO (w/v%), 4.4 g/L MS media salts and 0.4 M sucrose for 20 min.

Then half of the treated cryovials were plunged directly in LN for at least 2 hrs., while the other half was not frozen with LN and it was unloaded with unloading solution (MS media with 1.2 M sucrose) to remove PVS2. Next, the STs were inoculated into a recovery media (hormone free MS media plus 0.1 M sucrose), and stored under dark conditions for 1 week before being transferred to the normal growth conditions for 1 week. After four weeks, the STs were examined for any regrowth signs to growth room condition. For the cryopreserved STs, the STs were thawed for 2-3 min at 37-38 ºC. Then unloaded by exposing the STs to MS liquid media supplemented with (1.2 M) sucrose, then transferred to the recovery media and incubated under dark conditions for 1 week then transferred to the normal growth conditions. After four weeks, the STs were examined for any regrowth sign. For determination of survival percentage, STs from each of none cryopreserved (-LN) and cryopreserved with liquid nitrogen (+LN) were examined using of 2, 3, 5-Triphenyl Tetrazolium Chloride (TTC) assay and survival percentages of the STs were determined according to the following equation: Survival percentage = (number of red shoots /total number of shoots) ×100%

Effect Plant Vitrification Solution Type

Fennel STs were precultured in preculture media for 3 days under dark. Next, the precultured STs were loaded with 1 ml either of different loading solution types consisted of [10% DMSO and 0.75 M sucrose in HF- liquid MS media], [10% DMSO and 0.5 M sucrose in HF- liquid MS media] or [2 M Glycerol+ 0.4M sucrose in HF- liquid MS media] at room temperature for 20 min. Then each loading solution type was replaced by 1 ml of either of the following plant vitrification solutions:[15% DMSO and 1 M sucrose in HF- liquid MS media], [30% DMSO and 1 M sucrose in HF- liquid MS media], [PVS3 solution consisted of 40% (w/v) glycerol and 40% (w/v) sucrose in HF- liquid MS media] or PVS2, prepared as earlier. The STs were incubated at room temperature for 20 min.

Then half of the treated cryovials were plunged directly in +LN for at least 1 hr., while the other half was left -LN and unloaded with he unloading solution (MS media with 1.2 M sucrose) three times to wash out each PVS solution three times. After that, the STs were transferred to recovery media, and kept under dark conditions for 1 week, and subsequently transferred to the normal growth conditions for 1 week. After four weeks, the STs were examined for any regrowth signs at room condition. For the cryopreserved STs, the STs were thawed for 2-3 min at 37-38 ºC. Then unloaded as described earlier, then transferred to recovery media, and incubated as described above. The viability test of the treated STs was performed as described earlier.

2.2.2. Encapsulation-Dehydration

To encapsulate the STs inside the beads, two liquid media were prepared. The first medium consisted of 3% sodium alginate plus calcium free-MS salts. The second medium included MS media with 100 mM calcium chloride (CaCl2) and 0.3 M sucrose. Next, the STs were precultured into the preculture media, as described above and kept in dark for 3 days. After that, STs were taken individually with some alginate solution, and then soaked into liquid MS medium provided with 100 mM CaCl2 and 0.3 M sucrose to produce the beads and then the beads polymerized for 30 min with stirring.

Next, the resulted beads were transferred into MS liquid medium containing 0.5 or 0.75 M sucrose and rotated on a shaker for 1 or 3 days. After that, the media containing 0.5 M or 0.75 M sucrose was removed, and then the beads were air dehydrated under laminar air-flow cabinet for 0, 2, 4, and 6 hrs. Half of the beads were then transferred to 2 ml sterile cryovials and dipped into LN for at least 3 hrs. Next, the cryovials were rapidly thawed, while the other half of beads were not exposed to the cryogenic treatment. All STs were transferred to the recovery media and incubated, as described above. For the determination of beads moisture content, fresh weight of beads was measured after each dehydration period; then beads were dried at 80 ºC in an oven for 16 hrs and then reweighed.

Moisture Content (MC) was calculated using the following formula:

MC % = [(Beads fresh weight - Beads dry weight) / Beads fresh weight] ×100.

2.3. Experimental Design and Data Analysis

The treatments in each experiment described above were arranged in a Completely Randomized Design (CRD), and each treatment was replicated four times with five explants/ replicate. The collected data were statistically analyzed using Statistical Package for Social Sciences (SPSS version 20.0 for windows analysis system). The means and the standard errors for all experimental groups were calculated.

3. Results and Discussion

3.1. Vitrification

Effect of Loading Solution Combination on F. Vulgare Shoot Tips Survival and Regrowth

The obtained results showed that survival and regrowth rates varied with the type of the loading solution combination. For example, in none cryopreserved (-LN) shoot tips, full rates of survival

and regrowth were obtained in all treatments except in STs pretreated with either [10% DMSO + 0.25M sucrose] or [10% DMSO + 0. 5M sucrose] before exposure to PVS2 as they recorded (75 and 65%) survival and (45 and 25%) regrowth, respectively (Table 1). This might be a result of doubling DMSO concentration in the loading solution up to 10%, which might resulted in increasing sensitivity of the plant tissues to this highly toxic compound. Moreover, the exposure to PVS2 itself is a risky process in which STs suffer of being exposed to viscous hyperconcentrated solution. Meanwhile, the variation in survival and regrowth rates were more obvious in the cryopreserved STs (Table 1). The maximum survival (80%) and regrowth (65 and 60%) rates were recorded in STs preloaded with either (2M glycerol + 0.4M sucrose) or (5% DMSO + 0.5M sucrose). In many related studies, a combination of 2 M glycerol and 0.4 M sucrose was routinely used as a loading solution due to the high recovery rates obtained in many plant species after cryogenic exposure. Indeed, this combination described as the most popular loading solution type (Sakai and Engelmann, 2007). High recovery rates were also obtained in many wild medicinal plants in Jordan, such as mint, crocus, felty Germander, Shih and Qaysūm by using 2 M glycerol + 0.4 M sucrose as a loading solution before cryopreservation (Baghdadi *et al*., 2011; Rabba *et al*., 2012; Sharaf *et al*., 2012; Younis, 2012; Al- Baba *et al*., 2015). Also, in the present study using low concentration of DMSO (5%) in the loading solution might succeed in a supplementing wild fennel STs with a protective none toxic protection against PVS2 and LN shock which was translated into high survival and regrowth rates (80 and 60%) (Table 1).

Table 1. Survival and regrowth percentages of the non-cryopreserved (-LN) and cryopreserved with liquid nitrogen (+LN) wild fennel shoot tips as influenced by loading solution type

Cryoprotectant	Survival%	Regrowth%
Non- cryopreserved (-LN)		
1M sucrose	100± 0.0*	100± 0.0
2M glycerol + 0.4M sucrose	100± 0.0	100± 0.0
5% DMSO + 0.25M sucrose	100± 0.0	100± 0.0
5% DMSO + 0.5M sucrose	100± 0.0	95± 3.2
10% DMSO + 0.25M sucrose	75± 9.3	45± 3.8
10% DMSO + 0.5 M sucrose	65± 6.9	25± 11.4
Cryopreserved (+LN)		
1M sucrose	0.0± 0.0	0.0± 0.0
2M glycerol + 0.4M sucrose	80± 4.3	65± 5.3
5% DMSO + 0.25M sucrose	45± 6.8	0.0± 0.0
5% DMSO + 0.5M sucrose	80± 6.1	60± 7.1
10% DMSO + 0.25M sucrose	0.0± 0.0	0.0± 0.0
10% DMSO + 0.5 M sucrose	0.0± 0.0	0.0± 0.0

* values represent means ± standard error

Effect of the Loading Solution and Vitrification Solution Types

Data revealed that survival and regrowth rates of wild fennel STs were determined by types of loading and plant vitrification solutions (Tables 2, 3, 4). Full survival and regrowth rates were recorded when none cryopreserved STs of wild fennel were preloaded with (2M glycerol+ 0.4M sucrose) followed by 20 min exposure to either PVS2 or (15%DMSO+ 1M sucrose) (Table 2). A similar trend was obtained after cryopreservation, as the highest survival and regrowth rates (85 and 60%) were obtained in STs pretreated with (2M glycerol+ 0.4M sucrose) and PVS2 (Table 2). On the other hand, both rates were adversely affected in (-LN) and (+LN) STs when higher concentration of DMSO (30%) was used in the plant vitrification solution, which indicated that DMSO was very toxic at this level to plant tissues (Table 2). Meanwhile, exposing the STs to PVS3 was most deleterious before and after cryopreservation, as shown in Table (2), which might refer to the high chemical toxicity of PVS3 which might be confounded with the insufficient pretreatment duration with the loading solution before exposure to PVS3 (Subaih *et al*., 2007). Also, our results showed that PVS2 was the best plant vitrification solution type in -LN and +LN STs. For example, the best survival and regrowth rates (70, 45 and 20, 5%) in (+LN) STs were obtained in explants cryoprotected with PVS2 after being loaded with either (10% DMSO+ 0.5M sucrose) or (10% DMSO+ 0.75M sucrose), respectively (Tables 3, 4). On the other hand, data recorded in (+STs) cryoprotected with (15% DMSO + 1 M sucrose) before cryopreservation indicated that this combination failed to be a proper cryoprotectant, and the resulted the chemical dehydration was not enough to prevent ice crystallization and cryogenic injury (Sakai and Engelmann, 2007).

Meanwhile, all STs died after exposure to LN when pretreated with the other PVS combinations (Tables 3, 4), which indicated failure of these treatment to overcome the hazards of chemical toxicity and/or the cryogenic injury (Shatnawi *et al*., 2011). This was in full agreement with Markovic *et al.* (2013) who reported maximum regrowth percentages when grapevine STs exposed to PVS2 before cryopreservation. The same study showed the complete death of grapevine STs exposed to PVS3, which indicates the high toxic nature of PVS3.

Table 2: Survival and regrowth percentages of the non-cryopreserved (-LN) and cryopreserved with liquid nitrogen (+LN) shoot tips of wild fennel as influenced by vitrification solution combination

Loading solution type	Vitrification solution type	Survival %	Regrowth %
Non- cryopreserved (-LN)			
2M glycerol+ 0.4M sucrose	PVS2	100± 0.0	100± 0.0
	30% DMSO+ 1M sucrose	60± 9.1	40± 8.5
	15% DMSO+ 1M sucrose	100± 0.0	100± 0.0
	PVS3	50± 6.6	15± 7.3
Cryopreserved (+LN)			
2M glycerol+ 0.4M sucrose	PVS2	85± 4.3	60± 6.7
	30%DMSO+ 1M sucrose	20± 8.4	0± 0.0
	15%DMSO+ 1M sucrose	70± 5.1	55± 6.1
	PVS3	0± 0.0	0± 0.0

* values represent means ± standard error

Table 3: Survival and regrowth percentages of the non-cryopreserved (-LN) and cryopreserved with liquid nitrogen (+LN) shoot tips of wild fennel different vitrification solution combinations

Loading solution type	Vitrification solution type	Survival %	Regrowth %
Non- cryopreserved (-LN)			
10% DMSO+ 0.5M sucrose	PVS2	100± 0.0	100± 0.0
	30%DMSO+ 1M sucrose	30± 7.1	5± 7.8
	15%DMSO+ 1M sucrose	90± 4.3	80± 4.3
	PVS3	40± 6.7	0± 0.0
Cryopreserved (+LN)			
10% DMSO+ 0.5 M sucrose	PVS2	70± 5.4	20± 8.0
	30%DMSO+ 1M sucrose	0± 0.0	0± 0.0
	15%DMSO+ 1M sucrose	30± 7.4	0± 0.0
	PVS3	0± 0.0	0± 0.0

* values represent means ± standard error

Table 4: Survival and regrowth percentages of the non-cryopreserved (-LN) and cryopreserved with liquid nitrogen (+LN) shoot tips of wild fennel different vitrification solution combinations

Loading solution type	Vitrification solution type	Survival %	Regrowth %
Non- cryopreserved (-LN)			
10% DMSO+ 0.75M sucrose	PVS2	90± 3.2	80± 3.6
	30%DMSO+ 1M sucrose	25± 5.3	0±0.0
	15%DMSO+ 1M sucrose	70± 4.2	55± 4.7
	PVS3	15± 6.1	0±0.0
Cryopreserved (+LN)			
10% DMSO+ 0.75M sucrose	PVS2	45± 4.3	5± 6.7
	30%DMSO+ 1M sucrose	0± 0.0	0±0.0
	15%DMSO+ 1M sucrose	5± 6.2	0±0.0
	PVS3	0± 0.0	0±0.0

* values represent means ± standard error

3.2. Encapsulation Dehydration

The obtained results showed that regrowth rate of the encapsulated beads was determined by sucrose concentration, chemical incubation duration, air dehydration duration and bead moisture content after air dehydration. Also, it was obvious from the data that bead moisture content decreased with increasing sucrose concentration, chemical incubation and air dehydration durations (Tables 5-8). This in turn had a negative effect on regrowth rates of the encapsulated STs in (-LN) samples (Tables 5-8). However, after cryopreservation, the obtained regrowth results were completely opposite to those recorded in the (-LN) STs. For example, full death rates were recorded in all the encapsulated (+LN) STs with moisture content above (33.1 %) (Table 5-8). This might be a result of lethal ice crystallization which was formed due to the presence of freezable cellular water that indicates inadequate dehydration. Also, a complete death prevailed in STs with MC% less than (18.8 %) (Tables 5-8) which indicated that the STs were exposed to over desiccation due to preculture in high sucrose levels and/ or prolonged air dehydration durations. Hence, it might be very damaging to plant cells even before exposure to LN (Englemann, 2011).

Moreover, the best regrowth rate (65%) was recorded in wild fennel STs in +LN with 21.1% MC obtained after chemical dehydration in MS liquid media supplemented with (0.75 M) sucrose for 1 day followed by air dehydration for 6 hrs (Figure 1, Table 6), which completely agrees with (Shatnawi *et al*., 2011), as best regrowth rate was recorded in *Capparis spinosa* STs exposed to similar treatment before cryopreservation. Removal of the extracellular water and increasing osmotic potential inside the plant cells are the main goal of all pretreatment protocols applied before cryopreservation in order to avoid the hazard of ice crystallization upon exposure to LN. However, most plant species were reported to withstand the cryogenic exposure at bead moisture content range of (17- 37 %) (Englemann, 2011), which agrees to a certain extent with our results, as regrowth was recorded only in beads with MC% ranged from 33.1-18.8% (Tables 5-8). However, the obtained range indicated that it is specific to wild fennel, as optimum MC% was always reported to be dependent on plant species (Englemann, 2011). Examples on other related researches that agree with wild fennel results are those reported by Marco-Mediana *et al.* (2010), as (22%) was the optimum MC% for the cryopreserved STs of *Thymus moroderi*, while Markovic *et al.* (2013) indicated that 22.28% MC % was best for the encapsulated +LN STs of grapevine.

Table 5: Regrowth percentages of encapsulated dehydrated shoot tips of fennel as affected by dehydration duration after pretreatment with 0.5 M sucrose concentration for 1 day

Sucrose conc. (M)	Dehydration duration (hr)	Regrowth %	MC%
Non- cryopreserved encapsulated shoots tips (-LN)			
0.5M	0	100± 0.0	82.4
	3	100± 0.0	65.2
	6	100± 0.0	33.1
	9	65± 4.0	21.6
Cryopreserved encapsulated shoots tips with liquid nitrogen (+LN)			
0.5M	0	0± 0.0	82.4
	3	0± 0.0	65.2
	6	25± 5.6	33.1
	9	55± 4.3	21.6

* values represent means ± standard error

Table 6: Regrowth percentages of encapsulated dehydrated shoot tips of fennel as affected by dehydration duration after pretreatment with 0.75 M sucrose concentration for 1 day

Sucrose conc. (M)	Dehydration duration (hr)	Regrowth %	MC%
Non- cryopreserved encapsulated shoots tips (-LN)			
0.75M	0	100± 0.0	60.3
	3	100± 0.0	31.6
	6	85± 2.2	20.1
	9	40± 6.1	18.8
Cryopreserved encapsulated shoots tips with liquid nitrogen (+LN)			
0.75M	0	0± 0.0	60.3
	3	30± 6.7	31.6
	6	65± 4.2	20.1
	9	5± 8.1	18.8

* values represent means ± standard error

Table 7: Regrowth percentages of encapsulated dehydrated shoot tips of fennel as affected by dehydration duration after pretreatment with 0.5 M sucrose concentration for 3 days

Sucrose conc. (M)	Dehydration duration (hr)	Regrowth %	MC%
Non- cryopreserved encapsulated shoots tips (-LN)			
0. 5M	0	100.0± 0.0	65.6
	3	100.0± 0.0	33.5
	6	85.0± 3.0	22.1
	9	50.0± 4.8	19.2
Cryopreserved encapsulated shoots tips with liquid nitrogen (+LN)			
0. 5M	0	0.0± 0.0	65.6
	3	15± 6.4	33.5
	6	55± 4.4	22.1
	9	100± 0.0	19.2

* values represent means ± standard error

Table 8: Regrowth percentages of encapsulated dehydrated shoot tips of fennel as affected by dehydration duration after pretreatment with 0.75 M sucrose concentration for 3 days

Sucrose conc. (M)	Dehydration duration (hr)	Regrowth %	MC%
Non- cryopreserved encapsulated shoots tips (-LN)			
0.75M	0	100± 0.0	58.3
	3	100± 0.0	29.7
	6	40± 6.0	18.1
	9	10± 7.4	17.8
Cryopreserved encapsulated shoots tips with liquid nitrogen (+LN)			
0.75M	0	0± 0.0	58.3
	3	35± 4.0	29.7
	6	0± 0.0	18.1
	9	0± 0.0	17.8

* values represent means ± standard error

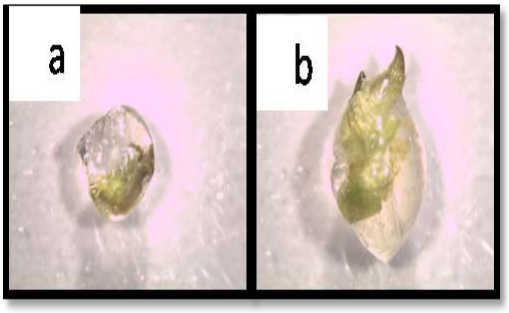


Figure 1: Encapsulated shoot tips of *F. vulgare* chemically dehydrated in MS liquid media supplemented with (0.75 M) sucrose for 1 day followed by air dehydration for 6 hrs. a: Directly after exposure to liquid nitrogen (LN). b: Two weeks after exposure to LN)

4. Conclusion

The obtained results indicated that long term conservation of wild fennel is possible. The results in both cryopreservation techniques (vitrification and encapsulation- dehydration) were very encouraging. However, more research is still needed to optimize the pretreatments protocols prior exposure to LN in terms of ingredients, concentration and duration to improve regrowth rates.

Acknowledgment

Authors would like to thank the University of Jordan, The Deanship of Academic Research, for granting fund for the present study. Further thanks are extended to Hamdi Mango Center for Scientific Research, the Faculty of Agriculture: the Department of Horticulture and Crop Sciences at the University of Jordan.

References

Al- Baba H, Shibli RA, Akash M , Al-Qudah T, Tahtamouni RW and Al- Ruwaiei H 2015. Cryopreservation and Genetic Stability Assessment of Threatened Medicinal Plant (*Ziziphora tenuior* L.) Grown Wild in Jordan. JJBS, **8**: 247 – 256.

Baghdadi S, Shibli RA, Syouf M, Shatnawi M, Arabiat A and Makhadmeh I. 2011. Cryopreservation by vitrification of embryogenic callus of wild crocus (*Crocus hyemalis* and *Crocus moabiticus*). *Acta Horticulturae*, **908**: 239-246.

Engelmann F. 2004. Plant cryopreservation: progress and prospects. *In Vitro Cell.Dev.Biol.-Plant*, **40**: 427- 433.

Engelmann F. 2011. Encapsulation – dehydration for cryopreservation: Past, present and future. *Acta Hor*, **908**: 165-170.

Fabre J. and Dereuddre J. 1990. Encapsulation-dehydration: A new approach to cryopreservation of Solanum shoot tips. *CryoLetters*, **11**: 413-126.

Ghazanfar SA, Taifour H, Al-Oqlah A, Borosova R and Osborne J. 2015. *Checklist of the Vascular Plants of Jordan*. Kew Publishing, London.

Gulfraz M, Mehmood S, Minhas N, Jabeen N, Kausar R, Jabeen K and Arshad G. 2008. Composition and antimicrobial properties of essential oil of *Foeniculum vulgare*. AJB, **7**: 4364-4368.

He W. and Huang B. 2011. A review of chemistry and bioactivities of a medicinal spice: *Foeniculum vulgare*. JMPR, **5:** 3595-3600.

Kasagana VN and Karumuri SS. 2011. Conservation of medicinal plants (past, present & future trends). JPS*R*, **3:**1378-1386.

Marcco-Medina A, Casas JL, Swnnen R and Panis B. 2010. Cryopreservation of *Thymus moroderi* by droplet vitrification. *CryoLetters*, **13:** 14-21.

Markovic Z, Chatelet P, Sylvertre I, Kontic JK and Engelmann F. 2013. Cryopreservation of grapevine (*Vitis vinifera* L.) *in vitro* shoot tips. CENT EUR J BIOL, **8:** 993-1000.

Mikuła A, Tomiczak K and Rybczyn´ski JJ. 2011. Cryopreservation enhances embryogenic capacity of Gentianacruciata (L.) suspension culture and maintains (epi)geneticuniformity of regenerants. PLANT CELL REP, **30:** 565-574.

Murashige T. and Skoog F. 1962. A revised media for rapid growth and bioassays with tobacco tissue cultures. PHYSIOL PLANTARUM, **15**: 473-479.

Rabba'a M, Shibli RA and Shatnawi M A. 2012. *In vitro* medium term conservation of felty germander *(Teucrium Polium L.)* micro-shoots. JJAS, **8 :** 523-544.

Raffo A, Nicoli S and Leclercq C. 2011. Quantification of estragole in fennel herbal teas: Implications on the assessment of dietary exposure to estragole. *Food Chem. Toxicol*, **49:** 370-375.

Rao NK. 2004. Plant genetic resources: Advancing conservation and use through biotechnology. AJB, **3:** 136-145

Royal Botanic Garden. (www.royalbotanicgarden.org.). **Cited on 5-10-2015.**

Sakai A and Engelmann F. 2007. Vitrification, encapsulation- vitrification and droplet- vitrification: A review. *CryoLetters***, 28 :** 151-172.

Sharaf SA, Shibli RA, Kasrawi MA and Baghdadi SH. 2012. Cryopreservation of wild shih (*Artemisia herba-alba*) Asso. shoot-tips by encapsulation-dehydration and encapsulation-vitrification. PCTOC, **108:** 437-444.

Shatnawi M, Shibli R, Al-Dmoor H, Ateyat M and Abubaker S. 2011. *In vitro* propagation and cryopreservation of *Stevia rebudian* using vitrification method**.** *Acta Horticulturae*, **908:** 319-324.

Shibli R, Shatnawi M, Subaih W and Ajlouni M. 2006. *In vitro* conservation and cryopreservation of plant genetic resources: a review. WJAS, **2:** 372-382.

Subaih WS, Shatnawi MA and Shibli RA. 2007. Cryopreservation of date palm (*Phoenix dactylifera*) embryogenic callus by encapsulation-dehydration, vitrification and encapsulation-vitrification. JJAS, **3:**156-171.

Tahtamouni R, Shibli R, Al- Abdallat A, Al-Qudah T, Younis L, Al- Baba H and Al- Ruwaiei H. 2015. *In Vitro* Conservation and Cryopreservation of Medicinal and Aromatic Plants: A Review. JJAS, **11**: 147-167.

Younes L. 2012. *In vitro* multiplication and conservation of *Achillea fragrantissima* Forssk. Sch. Bip. Ph.D. Dissertation. College of Agriculture, University of Jordan, Amman, Jordan.

JJBS

Volume 9, Number 3, September .2016
ISSN 1995-6673
Pages 155 - 161

Jordan Journal of Biological Sciences

Oxidation and Enzyme-activated Irreversible Inhibition of Rat and Ox Liver Mitochondrial Monoamine Oxidase-B by 2-(Benzylamino) Acetamide

Zakia M. Ben Ramadan[1*] and Keith F.Tipton[2]

[1]*Department of Biochemistry, Faculty of Medicine University of Tripoli-Libya*

[2]*Department of Biochemistry, Trinity College, Dublin 2, Irelan*

Received: May 16, 2016 Revised: July 9, 2016 Accepted: July 18, 2016

Abstract

The interactions of the anticonvulsant 2-n-pentylaminoacetamideanalogue, 2-benzylamino acetamide (FCE 25692) with MAO-B from rat and Ox liver mitochondria have been studied. This compound involves retention of the aminoacetamide portion of the parent compound but replacement of the pentyl moiety with benzylamine which is a good substrate for MAO-B. The results indicated FCE 25692 to be a good substrate for MAO-B from both preparations used with apparent K_m values of 229.8 and 920.0 μM and V_{max} values of 0.230 and 0.989nMol.min^{-1}.mg^{-1} for rat and ox liver mitochondrial MAO-B, respectively. It also acts as a suicide substrate and is a better substrate than it is an inhibitor for MAO-B from both species, with partitions ratios of 816.7 and 2120 mol of product per mol of enzyme inactivated, respectively for rat and ox liver mitochondrial MAOB. The partition ratio for ox liver MAO-B was considerably higher than that of the enzyme from rat liver and the half-life ($t_{1/2}$) of ox liver MAOB was a little larger than its respective ($t_{1/2}$) value for the enzyme from rat liver. The turnover numbers (k_{cat}) and the k_{cat}/K_m values are compared with the inhibition specificity constants (K_{in} /K') these values confirmed the fact that FCE 25692 is a better substrate for rat liver MAO-B than for ox liver MAO-B with kcat/Km values of (118 and 46.1 min^{-1}.mM^{-1}, respectively). While the inactivation constant k_{in} values showed that FCE 25692 is somewhat a better inhibitor for ox liver MAO-B than rat liver MAO-B (0.020 and 0.033, respectively). However the progress curves for the inhibition of MAO-B from both preparations showed that FCE 25692 was a better inhibitor of MAOB from rat preparation than ox preparation.

Keywords: Monoamine Oxidase-B (MAO-B), 2-(benzylamino) acetamide [FCE 25692], Suicide Substrate, Kinetic Parameters.

1. Introduction

Monoamine oxidase (MAO) (EC 1.4.3.4.) is a Flavin-Adenosine-Dinucleotide (FAD)-containing enzyme, (Tipton et al., 2004), associated with the outer membrane of the mitochondriain all mammalian cell types, with the notable exception of the erythrocyte. It converts biogenic amines to their corresponding aldehydes by oxidative deamination according to the following reaction:

$$RCH_2NH + O_2 + H_2O \rightarrow RCHO + NH_3 + H_2O_2$$

Its primary role lies in the metabolism of amines and in the regulation of neurotransmitter levels and intracellular amine stores. Because of the vital role that MAOs play in the inactivation of neurotransmitters, MAO dysfunction is thought to be responsible for a number of psychiatric and neurological disorders for example, unusually high or low levels of MAOs in the body have been associated with schizophrenia (Domino *et al*., 1976*;* Schildkraut *et al.,* 1976) and depression (Meyer *et al*., 2006).

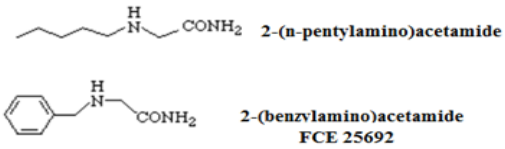
In humans there are two types of Monoamine oxidase, MAO-A and MAO-B. MAO-A is found in both dopaminergic, as well as noradrenergic neurons (Shih *et al*., 2004) and may help eliminate 5-HT in these neurons. Similarly, MAO-B found in serotonergic neurons may protect these cells from the entry of dopamine and2-phenylethylamine (PEA), for which MAO-B shows a preferred affinity (Tipton *et al*., 1975). MAO's role in limiting the presence of these neurotransmitters following their release may secondarily result in altering the release of hormones from cells sensitive to biogenic amines. Thus, MAO may function indirectly as a regulator of neuroendocrine function.

Monoamine oxidase is a well-known enzyme in pharmacology, since it is the substrate for the action of a number of monoamine oxidase inhibitor drugs. In fact, MAO-A inhibitors

* Corresponding author. e-mail: *Zakia.ramadan@gmail.com*

act as antidepressant and antianxiety agents, whereas MAO-B inhibitors are used alone or in combination to treat Alzheimer's and Parkinson's diseases (Riederer *et al.*, 2004), although they are often last-line treatment due to risk of the drug's interaction with diet or other drugs.

The discovery that the antidepressant drug iproniazid (1-isonicotinoyl-2-isopropylhydrazine) was an inhibitor of monoamine oxidase and it belongs to hydrazines, the first known suicide (mechanism-based) enzyme inhibitors of MAO that act as enzyme activated irreversible inhibitors where the active inhibitory species is formed through the action of MAO itself, stimulated the development of many other inhibitors of this enzyme, which have been used as antidepressants, for review see (Tipton, 1989; Ben Ramadan *et al*., 2007; Shulman *et al*., 2013). The benzylamine derivative 2-benzylamino acetamide, also designated as:



FCE 25692, belongs to a series of analogues of milacemide (2-n-pentylamino acetamide) that acts as both a 'suicide' substrate and specific MAO-B inhibitor (Dostert *et al*., 1990), a glycine derivative with atypicalanti-epileptic and potential psychotropic properties acts as a pro-drug delivering glycine into the central nervous system. Janssens de Varebeke *et al*. (1988 & 1989) first suggested that the capability of brain MAO-B to metabolize milacemide to glycinamide with a concomitant increase of brain glycine may be a prerequisite for milacemide'santiconvulsant activity, but it now seems apparent that the 'delivery of glycine to the brain' hypothesis for anticonvulsant activity remains an imperfect model. Despite the doubt on the role of glycine formation in the anticonvulsant actions of milacemide, the possibility of using derivatives of this compound for the delivery of pharmacologically-activecompounds to the brain remains. In the present work comparative kinetic studies on the behavior of 2-benzylaminoacetamideas a substrate and an inhibitor of MAO-B from rat and ox liver mitochondrial preparations have been done. FCE 25692 was reported by Dostert *et al*. (1991), to be a potent and selective rat liver MAO-B inhibitor (IC_{50} = 60 nM, without enzyme-inhibitor preincubation) and to be deaminated at a slow rate as compared with milacemide and some of the other analogues. Furthermore, it displayed little protection against tonic convulsions and death in mice.

2. Experimental Procedures

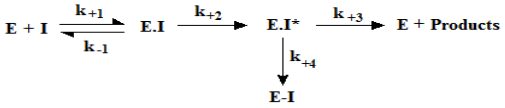
2.1. Materials

Benzylamine HCL was obtained from Sigma Co. (2-(benzylamino) acetamide) FCE 25692 was synthesized at Farmitalia Carlo Erba, Milan, Italy. All other chemicals were standard laboratory chemicals and were of analytical reagent grade whenever possible.

2.2. Methods

Rat liver mitochondria were prepared by the method of Kearney et al. (1971). The mitochondrial pellet obtained was suspended in a small volume of 0.1M potassium phosphate buffer, pH 7.2 and stored at -20oC until use for MAO-B. Ox liver mitochondria were prepared by the method of Salach (1979). All enzyme assays were performed at 37oC and pH 7.2. MAO-B activity was determined spectrophotometrically by directly monitoring the formation of benzaldehyde from benzylamine by following the increase in absorbance at 250 nm Tabor et al. (1954). The reaction mixture contained 93 mM potassium phosphate buffer, pH 7.2, enzyme preparation at the indicated concentrations and (333 µM) benzylamine. The molar extinction coefficient ( ) of benzaldehyde at 250 nm was taken to be 13.8 x 103 M-1.cm-1 (Tipton & Youdim, 1983). The oxidation of the suicide substrate FCE 25692 by MAO was examined by the direct spectrophotometric assay for benzaldehyde formation at 250 nm. The Kinetic constants Vmaxand Km were determined using the computer program ENZFITTER. The double reciprocal plots are used only for illustrative purposes. The reaction progress curves were analyzed using the computer program MACCURVE-FIT to estimate the values of the maximum product formation at time = ∞ (Amax) and the apparent first-order rate constant for the decline in activity with time (kapp).

The partition ratio (r), which represents the number of mol of product formed per mol of enzyme inhibited (or k3/k4)was calculated by determining the amount of product formed at complete inactivation [P∞] for different enzyme inhibitor ratios, according to the relationship (Waley, 1980) $r = (k+3 / k+4) = [P_\infty] / e_0$



(I) FCE 25692, (E) the Enzyme, (E.I) a non-covalent compound, (EI*) an activated intermediate, (E-I) the irreversibly inhibited species

The direct spectrophotometric assay for aldehyde formation was used to examine the reactions of MAO-B (at different concentrations) with FCE 25692 (at different concentrations) while enzyme: FCE 25692 ratios were kept constant. An Uvikon-931 double beam spectrophotometer equipped with a multicellauto changer, which allow the sequential determination of six samples with the appropriate blanks where the temperature was controlled by the use of a circulating water bath was used. The curves were analyzed by the procedure of Waley (1980 & 1985), using the MACCURVE-FIT

computer program. Then the apparent Km value (K') for the inhibition reaction and the inactivation constant (kin) for the inhibition process were determined according to the following relationships, Waley (1980 &1985):

$$I_o t_{1/2} = \left(\frac{\ln (2-M)}{(1-M)}\right) \frac{K'}{k_{in}} + \frac{\ln 2}{k_{in}} I_o$$

$$k_{in} = \frac{k_{+2}\ k_{+4}}{k_{+2} + k_{+3} + k_{+4}} = \frac{\ln 2}{\text{slope}}$$

$$K' = (\text{ordinate intercept}).k_{in} \frac{(1-M)}{\ln(2-M)}$$

where: $M = (1+r).e_o / I_o$

I_o and e_o are the initial inhibitor and enzyme concentration, respectively.

3. Results

3.1. Time-Courses of Oxidation of FCE 25692 by Liver Mitochondrial MAO-B from Both Species

The initial rates of FCE 25692 oxidation, were found to be linear function of the enzyme concentration. The time-courses for the oxidation of FCE 25692 by MAO-B from both species used deviated from linearity after few minutes of starting the reactions. After the reaction had ceased almost completely (55 and 100 min) for rat and ox liver MAO-B, respectively, it could not be restored by the addition of more substrate indicating that the reaction had not ceased due to substrate depletion or the establishment of an equilibrium of a reversible reaction. Neither could any activity be detected when (333µM) benzylamine was added. However, the addition of more enzymes was found to restore the activity as shown in Fig. 1 (it is taken as a representative for the enzyme from both preparations).

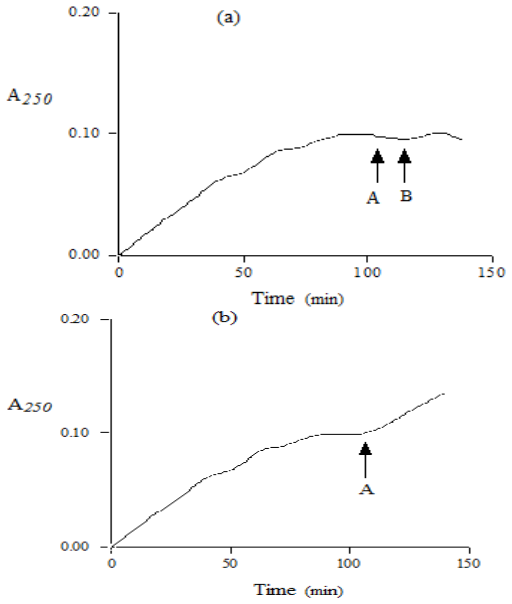


Figure 1. Time Course of Oxidation of FCE 25692 by Rat Liver Mitochondrial Monoamine Oxidase-B

Figs. 2 and 3 show series reaction progress curves of rat and ox liver mitochondrial MAO-B with different concentrations of FCE 25692, respectively. These reactions obeyed the Michaelis-Menten kinetics. The Michaelis constants (Km) and the maximum velocities (Vmax) for the oxidation of FCE 25692 by MAO-B from the enzyme preparations used were determined and compared with their respective values for the parent amine benzylamine. The values obtained are shown in (Table 1).

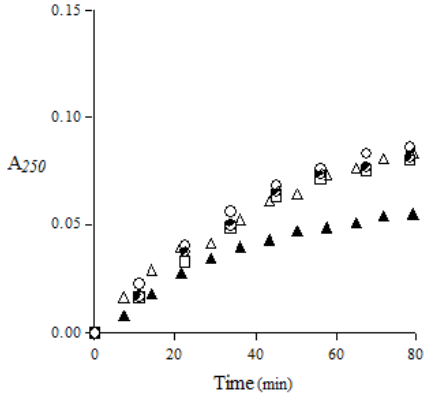


Figure 2. Time Courses of Oxidation of FCE 25692 at a Series of Different Concentrations by Rat Liver Mitochondrial MAO-B.The reactions between rat liver mitochondria (600 µg) and FCE 25692, (▲) 1, (△) 2, (□) 3, (◕) 4, and (⬭)5mM, were followed spectrophotometrically at 250 nm. using the direct assay. The points shown are the results from five representative experiments.

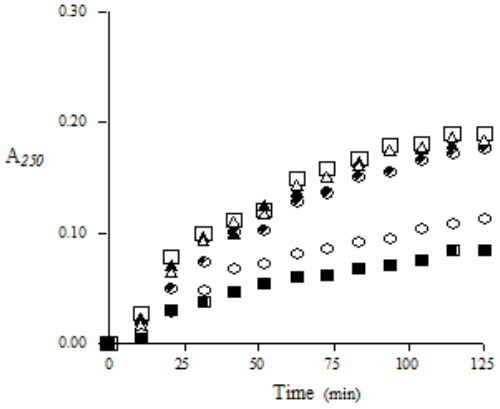


Figure 3. Time Courses of Oxidation of FCE 25692 at a Series of Different Concentrations by Ox Liver Mitochondrial MAO-B.

Table 1. The Kinetic Parameters for the Oxidation of FCE 25692 and Benzylamine by Monoamine Oxidase-B from the two Preparations

Enzyme preparation	Rat Liver Mitochondria	Ox Liver Mitochondria
Substrate	**Benzylamine FCE 25692A**	**Benzylamine FCE 25692A**
$\mathbf{K_m}$ (μM) (2)	211.8 ± 33.9 (3) 229.8 ± 10.0 (3)	144.4 ± 9.8 (2) 920.0 ± 15
$\mathbf{V_{max}}$ (nMol.min^{-1}.mg^{-1}.)	7.89 ± 0.424 0.230 ± 0.05	21.45 ± 3.2 0.989 ± 0.03

The Direct spectrophotometric assay at 250 nm was used in all cases. Values quoted are the means + standard errors from the curve fits obtained from two or more separate determinations (as shown in brackets).

Figs. 4 and 5 illustrate the determination of the Michaelis constants for the oxidation of FCE 25692 and the parent amine benzylamine by rat liver MAO-B, respectively, and are taken as being representative of the other enzyme form used. The progress curves for the inhibition of monoamine oxidase-B from both species used by FCE 25692 would be consistent with this compound acting as both a substrate and as an irreversible mechanism-based inhibitor of the enzyme (suicide substrate) according to the mechanism shown in Scheme-1.

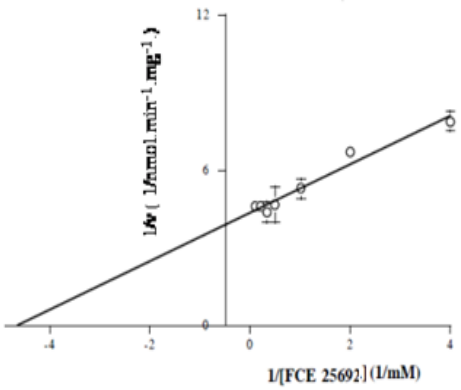


Figure 4. Determination of the Michaelis Constant for the Oxidation of FCE 25692 by Rat Liver Mitochondrial MAO-B.

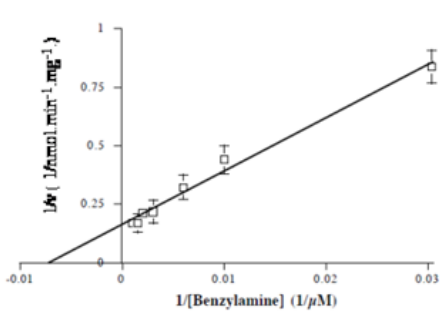
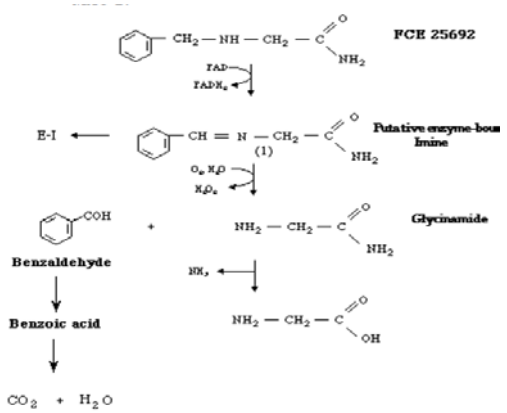


Figure 5. Determination of the Michaelis Constant for the Oxidation of Benzylamine by Rat Liver Mitochondrial MAO-B



Scheme 1. The mechanism of FCE 25692 oxidation by MAO-B as would be expected since FCE 25692 acts as both a 'suicide' substrate and specific MAO-B inhibitor. E-I represents the irreversibly inhibited species.

The partition ratio (r) was calculated by determining the amount of product formed at complete inactivation [P∞] for different enzyme inhibitor ratios. The absolute enzyme concentrations (eo) were determined as described in (Ben Ramadan *et al.*, 2012). The values obtained for the partition ratio of rat and ox liver mitochondrial MAO-B were 817 and 2120, respectively (Table 2.).

Table 2. The Kinetic Parameters for the interactions of the two Preparations of Monoamine Oxidase-B with FCE 25692.

Enzyme preparation	Rat Liver Mitochondria	Ox Liver Mitochondria
t1/2 (min)	21.5±3.5	34.0 ± 9
r_	816.7±121	2120 ± 55
K'(mM)	1.622±0.02	1.598± 0.01
Kin (min-1)	0.033±0.001	0.020±0.002

The values were determined by the procedure of Waley (1980 &1985) as described in the text. Each value represents the mean ± S.E.M. For at least 3 replicates

3.2. The Kinetic Parameters for the Mechanism Based Interactions of MAO-B from Both Species with FCE 25692

Figs. 6a and 7a show a series of reaction-progress curves for rat liver mitochondrial MAO-B and ox liver mitochondrial MAO-B, respectively with different concentrations of FCE 25692 but with the enzyme: FCE 25692 ratios kept constant. These curves were analyzed by the procedure of Waley (1980 &1985), as described earlier and a plot of $I_o.t_{1/2}$ versus the initial FCE 25692 concentration (I_o) were constructed for each (Figs.6b and 7b, respectively). From these the values of the apparent K_m (K') and the inactivation constants (k_{in}) obtained and they were (K') 1.60, and 1.62 mM and (k_{in}) 0.020, and 0.033 min^{-1}for ox liver mitochondrial MAO-B and rat liver mitochondrial MAO-B, respectively, as shown in Table 3.

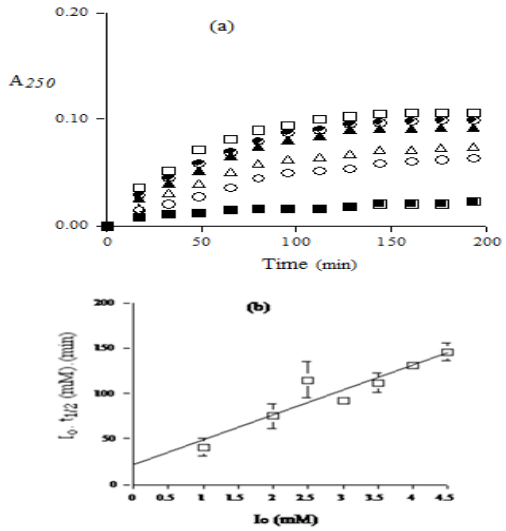


Figure 6. Determination of the Kinetic Parameters of Rat Liver Mitochondrial MAO-B towards FCE 25692.

a) Time Courses of Oxidation of FCE 25692 by Rat Liver Mitochondrial MAO-B at Different Concentrations of Each: The reactions of rat liver mitochondrial MAO-B and FCE 25692 (■) 1, (⬭) 2, (△) 2.5, (▲) 3, (◕) 3.5, and (□) 4 mM, were followed spectrophotometrically at 250 nm. The ratio between the enzyme concentration and FCE 25692 concentration was fixed. The points shown are the results from six representative experiments.

b) Half-time Plot for the Mechanism Based Inhibition of Rat liver Mitochondrial Monoamine oxidase-B by FCE 25692: The plot of $[I_o].t_{1/2}$ against $[I_o]$ for a series of experiments as shown above (a) in which $e_o/[I_o]$ was kept fixed where e_o is the initial concentration of rat liver mitochondrial MAO-B and I_o is the initial concentration of FCE 25692 .The points shown are the mean values ± range from two separate experiments.

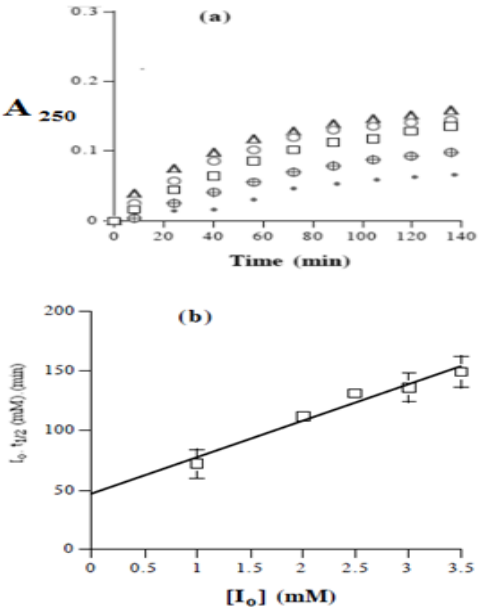


Figure 7. Determination of the Kinetic Parameters of Ox Liver Mitochondrial MAO-Btowards FCE 25692

a) Time Courses of Oxidation of FCE 25692 by Ox Liver Mitochondrial MAO-B at Different Concentrations of Each: The reactions of ox liver mitochondrial MAO-B and FCE 25692 , (*) 1, (⊕) 2, (□) 2.5, (⬭) 3, and (△) 3.5mM, were followed spectrophotometrically at 250 nm. The ratio between the enzyme concentration and FCE 25692 concentration was fixed. The points shown are the results from five representative experiments.

b) Half-time Plot for the Mechanism Based Inhibition of Ox liver Mitochondrial Monoamine oxidase-B by FCE 25692: The plot of [Io].t 1/2 against [Io] for a series of experiments as shown above (a) in which eo /[Io] was kept fixed where eo is the initial concentration of ox liver mitochondrial MAO-B and Io is the initial concentration of FCE 25692 . The points shown are the mean values ± range from two separate experiments.

Table 3. A Comparison of the kinetic Parameters for FCE 25692 as a Substrate and an Inhibitor of the Monoamine Oxidase-B Preparations

Enzyme preparation	Rat Liver Mitochondria	Ox Liver Mitochondria
Km (mM)	0.229	0.920
Kcat (min-1)	27.3	42.4
kcat/Km(min-1.mM-1)	118.7	46.1
K' (mM)	1.622	1.598
Kin (min-1)	0.033	0.020
kin/K' (min-1.M-1)	20.6	12.8

The catalytic constant kcat (maximum velocity / enzyme concentration) = Kin.r.

4. Discussion

The progress curves for the inhibition of MAO-B by FCE 25692 would be consistent with the compound acting as both a substrate and time-dependent irreversible inhibitor of the enzyme. This shows that the mechanism of action of FCE 25692 is similar to the mechanism of action of milacemide (Ben Ramadan et al., 2012) which acts as both a substrate and inhibitor of MAO-B and to that described for MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) (Tipton et al.,1986) and 3-{4-[(3-chlorophenyl)methoxy]phenyl}-5-[(methylamino)methyl]-2-oxazolidinone methanesulphonate (MD 780236) (Tipton et al., 1983a). The reaction pathway of FCE 25692 oxidation by MAO-B, would be expected as shown in Scheme-1. FCE 25692 is oxidized by MAO-B to form an imine intermediate. This intermediate can react with MAO-B to form a covalently bound intermediate that inactivates the enzyme or is hydrolyzed to liberate glycinamide and benzaldehyde. The latter product is then oxidized to benzoic acid. Benzoic acid is readily oxidized to carbon dioxide and water, whereas, glycinamide is broken down by amidase activities in brain microsomes to glycine. The detailed reaction mechanism may be more complex than this with intermediate radical intermediates being the actual inhibitory species. Unlike the behavior of milacemide (Ben Ramadan et al., 2012), the present

study did not show a large species differences between rat and ox liver MAO-B and the progress curves of the reactions, as monitored using the direct spectrophotometric assay, were seen to depart from linearity after few minutes with both MAO-B preparations studied. The values of the half-lives (Table 2) confirmed this; the t1/2value for ox preparation was a little larger than that for the enzyme from rat liver. The high values of the partition ratios Table (2) for the preparations used indicate that FCE 25692 functions as a better substrate for the enzyme MAO-B than as a mechanism-based inhibitor. In accordance with the behavior of milacemide (Ben Ramadan et al., 2012), the partition ratio for ox liver MAO-B (2120) is considerably higher than that of the enzyme from rat liver (817):

The progress curves for the inhibition of MAO-B from the two preparations showed that FCE 25692 was a better inhibitor for rat preparation and this is in agreement with the IC50 values previously reported by (O'Brien et al.,1994). FCE 25692 was shown to have a 4-fold lower Km value as a substrate for rat liver MAO-B (229 µM) than for the ox liver enzyme (920 µM). Thus, on these criteria, FCE 25692 is a better substrate for rat liver MAO-B than ox liver MAO-B. However, the kcat values (Table 3) obtained for mitochondrial enzymes from ox liver (42.0min-1) were somewhat higher than that for the rat liver mitochondrial MAO-B (27.3min-1). These differences are reflected in the specificity constants (kcat/Km values) which are the most useful indicators of the substrate specificity of an enzyme (see Cornish-Bowden, 1974). The turnover numbers (kcat) and the kcat/Km values are compared with the inhibition specificity constants (Kin /K') in Table 3. These values confirmed the fact that FCE 25692 is a better substrate for rat liver MAO-Bthan for ox liver MAO-B with kcat/Km values of (118 and 46.1 min-1.mM-1, respectively). Though the kinvalues were not much different, they show that FCE 25692 is somewhat a better inhibitor for ox liver MAO-B than rat liver MAO-B. The selectivity of FCE 25692 and milacemide as substrates for MAO-B may be related to the lipophilicity of these compounds, which is a common property of several MAO-B substrates. It is clear that milacemide is a much better substrate for MAO-B from rat and ox preparations (Ben Ramadan et al., 2012) than its benzylamine derivative, FCE 25692 in present studies. This could reflect the behavior of the precursor amines from which they were derived, since n-pentylamine, the parent amine of milacemide has a much lower Kmtowards MAO-B (Ben Ramadan et al., 2012) than benzylamine, the parent amine of FCE 25692; this difference may be an effect of the electronegative benzene ring of benzylamine. At low FCE 25692 concentrations the relative values of kin / K' (the inhibition specificity constant) for ox liver MAO-B was about half that for rat liver MAO-B. This is different than the inhibition specificity constants for milacemide (Ben Ramadan et al., 2012), where the value for the ox liver enzyme was found to be more than 6-times lower than that for the rat liver enzyme.

The reaction between rat liver mitochondria (600 µg) and FCE 25692 (2 mM) was followed spectrophotometrically at 250 nm using the direct assay. Upon completion of initial reaction, (a) at the point indicated by A, a further sample of FCE 25692 was added to raise the final concentration by 2 mM. In a second parallel experiment, at the point indicated by B (333µM) benzylamine was added. (b) At the point indicated by A more rat liver mitochondria was added to the reaction mixture and the reaction was followed further.

The initial rates of oxidation of FCE 25692 by rat liver mitochondria were measured, over the indicated range of concentrations. The results are presented as a double reciprocal Line weaver-Burk plot. Each point represents the mean value from three separate experiments.

The initial rates of oxidation of benzylamine by rat liver mitochondria were measured, over the indicated range of concentrations. The results are presented as a double reciprocal Line weaver-Burk plot. Each point represents the mean value from three separate experiments.

Acknowledgement

I am grateful to the staff in the biochemistry laboratory for their cooperation during my work at Trinity College Dublin as a research fellow.

References

Ben Ramadan Z, Tipton KF and Maria L. 2007. (N) - - Species Differences in the Selective Inhibition of Monoamine Oxidase (1-methyl-2-phenylethyl) hydrazine and its Potentiation by Cyanide. *Neurochem Res*, DOI 10.1007/s11064-007-9309-x.

Ben Ramadan Z and Tipton KF. 2012. Suicide Inhibition of Monoamine Oxidase from Different Species By Milacemide. *Jor J Biol Sci*, **5**: 269-278.

Cornish-Bowden A. 1974. Inhibitors and Activators in: Fundamentals of Enzyme Kinetics, (Cornish-Bowden, A., ed.), pp. 84-85, *Butterworths, London.*

Domino EF and Khanna SS. 1976. Decreased blood platelet MAO activity in unmedicated chronic schizophrenic patients. *The American Journal of Psychiatry*, **133**: 323-326.

Dostert P, Perarello P, Heidempergher F, Varasi M, Bonsignori A and Roncucci R. 1990. Preparation of a-(phenylalkylamino) carboxamides as drugs. *Eur Pat Appl*, **400**: 495.

Dostert P, Strolin Benedetti M and Tipton KF. 1991. New anticonvulsants with selective MAO-B inhibitoty activity. *Eur J Neuropsychopharmacol*, **1**: 317-319.

Janssens de Varebeke P, Cavalier R, David-Remacle M and Youdim MBH. 1988. Formation of the neurotransmitter glycine from the anticonvulsant milacemide is mediated by brain monoamine oxidase B. *J Neurochem*, **50**: 1011-1016.

Janssens de Varebeke P, Pauwells G, Buyse C, David-Remacle M, De Mey J, Roba J and Youdim MBH. 1989. The novel neuropsychotropic agent milacemide is a specific enzyme activated inhibitor of brain monoamine oxidase-B. *J Neurochem*, **53**: 1109-1116.

Kearney EB, Salach JI, Walker W H, Seng RL, Kenney W, Zeszotek E and Singer TP. 1971. The covalently bound flavin of hepatic monoamine oxidase. I. Isolation and sequence of a flavin peptide and evidence for binding at the 8a position. *Eur J Biochem*, **24:** 321-327.

MeyerJH, GinovartN,Boovariwala A,Sagrati S, HusseyD, Garcia A, Young T, Praschak-Rieder N, Wilson AA and Houle S . 2006. Elevated monoamine oxidase a levels in the brain: an explanation for the monoamine imbalance of major depression. *Arch Gen Psych*, **63**:1209–1216.

O'Brien EM, Dostert P, Paolo P and Tipton KF. 1994. Interactions of some analogues of the anti convulsant milacemide with monoamine oxidase. *Biochem Pharmacol*, **48**: 905-914.

Riederer P, LachenmayerLand Laux G. 2004.Clinical applications of MAO-inhibitors. *Curr Med Chem*, **11**: 2033–2043.

Salach JI. 1979. Monoamine oxidase from beef liver mitochondria: simplified isolation procedure, properties and determination of its cysteinylflavin content. *Arch Biochem Biophys*, **192**: 128-137.

Schildkraut JJ, Herzog JM, Orsulak PJ, Edelman SE, Shein HM and Frazier SH. 1976.Reduced platelet monoamine oxidase activity in a subgroup of schizophrenic patients. *Amer J Psych*, **133**: 438-440.

Shih JC and Chen K. 2004.Regulation of MAO-A and MAO-B gene expression. *Curr Med Chem*, **11**: 1995-2005.

Shulman KI, Herrmann N and Walker E S. 2013. Current Place of Monoamine Oxidase Inhibitors in the Treatment of Depression. *CNS Drugs*, **27**:789-797.

Tabor CW, Tabor H and Rosenthal SM. 1954. Purification of amine oxidase from beef plasma. *J Biol Chem*, **208**: 645-661.

Tipton KF. 1975. Monoamine oxidase in: Handbook of Physiology Section7: Endocrinology, vol. 6 (Blaschko, H., Sayers, G. & Smith, A.D., eds.), pp. 677-697, *American Physiological Society*, Washington.

Tipton KF and Youdim MBH. 1983. The assay of monoamine oxidase activity in: Methods in Biogenic Amine Research (Parvez, S., Nagatsu, T., Nagatsu, I. & Parvez, H., eds.), pp. 441-465, *Elsevier Press*, Amsterdam, New York and Oxford.

Tipton KF, Fowler CJ, McCrodden JM and StrolinBenedetti M. 1983a. The enzyme-activated irreversible inhibition of type-B monoamine oxidase by 3-(4-((3-chlorophenyl)methoxy) phenyl)-5((methylamino)methyl)-2-oxazolidinone methane sulphonate (compound MD 780236) and the enzyme-catalysed oxidation of this compound as competing reactions. *Biochem J*, **209**: 235-242.

Tipton KF, McCrodden JM and Youdim MBH. 1986. Oxidation and enzyme-activated irreversible inhibition of rat liver monoamine oxidase-B by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). *Biochem J*, **240**: 379-383.

Tipton KF. 1989. Monoamine oxidase inhibitors as antidepressants. In: Tipton KF and Youdim MBH. (eds) Biochemical and pharmacological aspects of depression. Taylor & Francis, London, pp 1–24.

Tipton KF, Boyce S, O'Sullivan J, DaveyGP and Healy J. 2004. Monoamine oxidases: certainties and uncertainties. *Curr Med Chem*, **11**: 1965-1982.

Waley SG. 1980. Kinetics of suicide substrates. *Biochem J*, **185**: 771-773.

Waley SG. 1985. Kinetics of suicide substrates. Practical procedures for determining parameters. *Biochem J*, **227**: 843-849.

JJBS

Volume 9, Number 3, September .2016
ISSN 1995-6673
Pages 163 - 171

Jordan Journal of Biological Sciences

Phytochemicals, Antioxidative and *in vivo* Hepatoprotective Potentials of *Litsea floribunda* (BL.) Gamble (Lauraceae) - An Endemic Tree Species of the Southern Western Ghats, India

Mruthunjaya Devika[1], Hanumanthachar Joshi[2], and Monnanda Somaiah Nalini[3*]

[1] *Department of Botany, Sarada Vilas College, Krishnamurthypuram, Mysore-570 004, Karnataka, India*

[2]*Division of Pharmacognosy, Department of Post-graduate Studies & Research, Sarada Vilas College of Pharmacy, Krishnamurthypuram, Mysore-570 004, Karnataka, India*

[3]*Department of Studies in Botany, University of Mysore, Manasagangotri, Mysore-570 006,Karnataka, India*

Received: February 16, 2016 Revised: June 17, 2016 Accepted: July 5, 2016

Abstract

The leaf and stem bark samples of *Litsea floribunda* (Bl.) Gamble a medicinal tree species, endemic to the Western Ghats, a biodiversity 'hot spot' of India were subjected to soxhlet extraction, phytochemical analysis and antioxidant activity by employing *in vitro* screening methods. The extracts exhibiting high antioxidant activity were assessed for the hepatoprotective activity against paracetamol-induced liver damage in rats. Phytochemical analysis of the stem bark and leaf extracts indicated the presence of flavonoids, terpenoids, cardiac glycosides, tannins, saponins and reducing sugars in the hexane, chloroform, ethyl acetate, absolute ethanol, methanol and aqueous extracts, respectively. The *in vitro* antioxidant activities revealed that the absolute ethanol and the aqueous extracts of stem bark as well as leaves exhibited high activity. The aqueous (98.9± 0.7 mg/g GAE), and absolute ethanol extracts (114.8±0.2 mg/ g GAE) of leaf as well as the stem bark aqueous (112.4±0.7 mg/g GAE) and absolute ethanol extracts (117.4±0.1 mg/g GAE) extracts contained higher amount of total phenolics compared to other solvent extracts. On the basis of the IC_{50} values (21.5±0.8 µg/ml to 29.0±0.2 µg/ml), the leaf/stem bark absolute ethanol and the aqueous extracts were considered for hepatoprotective studies in the *in vivo* rat model. The absolute ethanol and aqueous leaf and stem bark extracts were administered orally to 11 groups of animals in two doses (250 and 500 mg/kg b.w) with hepatotoxicity induced by paracetamol (2 mg/kg b.w.). Silymarin (100 mg/kg b.w.), the standard drug was used as a positive control. The stem bark aqueous and absolute ethanol extracts were effective in protecting the liver against the toxicity induced by paracetamol in rats. This was evident from the significant reduction in serum enzymes such as Serum Glutamic Pyruvate Transaminase (SGPT), Serum Glutamic Oxaloacetate Transaminase (SGOT), alkaline phosphatase (ALP), serum bilirubin, serum total proteins and liver weight against the treated group of animals. Histopathological studies conducted by sectioning of liver samples also indicated the hepatoprotective nature of the stem bark absolute ethanol and aqueous extracts with moderately dilated hepatic vein and degenerated peripheral hepatocyte infiltration suggestive of reduced toxicity. The results obtained strongly indicate that the absolute ethanolic and aqueous extracts of *L. floribunda* stem bark has good antioxidant activity

1. Introduction

The medicinal value of the plant lies in the bioactive phytochemical constituents, which work with nutrients and fibers to form an integrated part of defense system against stress conditions and diseases (Koche et al., 2010). Living cells generate free radicals and other reactive oxygen species as a result of physiological and biochemical processes. Oxidative damage to lipids, proteins and DNA by free radicals leads to chronic diseases. Plant constituents form an important source of antioxidants that are capable of scavenging free radicals and prevent them from cell and tissue damage and terminate the free radical chain reactions. The liver is the main site for intense metabolism and excretion. Liver diseases are a serious challenge to international public health (Yadav et al., 2011). Toxic chemicals, xenobiotics, alcohol consumption, malnutrition and medications cause liver damage due to exposure to high quantities of free radicals leading to oxidative stress (Alaqsoumi et al., 2014). Unfortunately, synthetic drugs used in the treatment of liver damage are inadequate and can have serious side effects. Therefore, there is a growing demand for the traditional herbal medicines that have hepatoprotective activity. In Ayurveda, 40 herbal formulations are used for hepatoprotection and

contain phenols, coumarins, essential oils, monoterpenes, carotenoids, flavonoids, alkaloids and glycosides (Sheik et al., 2012).

In recent years, there is a worldwide increase in the use of natural antioxidants present in herbs, fruits and vegetables as against the synthetic antioxidants. Antioxidants offer resistance against oxidative stress by scavenging free radicals and inhibiting lipid peroxidation. The property of hepatoprotection of plant extracts is through one of the mechanisms such as antioxidative, anti-lipid peroxidative, anti-fibrotic, immunomodulatory and liver-regenerating effects (Luper, 1998). The phenolics and flavonoids present in plant extracts attribute to antioxidant and hepatoprotective activity against liver injury (Aseervatham, 2013). Lauraceae, referred to as the laurel family, includes about 55 genera and 4000 species world-wide, mostly from warm or tropical regions, especially Southeast Asia and South America. The members are aromatic trees or shrubs forming laurel forests in the tropics. Litsea is a member of Lauraceae comprising ~200 species, in the tropical and subtropical Asia, Australia, North America and subtropical South America. 45 species are documented in India of which, 18 are endemic (Bhuniya et al., 2010a). In the traditional medicine, the leaf and bark of Litsea are used (Bhuniya et al., 2010b). In the Chinese medicinal system, it is used in the treatment of diarrhea, stomachache, dyspepsia, gastroenteritis, diabetes, edema, arthritis, pain etc. (Kong et al., 2015).

Litsea floribunda (Bl.) Gamble is an arborescent, endemic and predominant species of the shola vegetation in the Western Ghats, a biodiversity 'hotspot' of southern India. It is also documented from the coffee plantations and sacred groves (Bhagwat et al., 2005). The leaves of L. floribunda are used as one of the ingredients in the preparation of herbal shampoo, in southern India (Girish et al., 2014). In the health traditions, the local inhabitants use L. floribunda to treat certain gastrointestinal and respiratory disorders (personal observation). Till now, no data are available on the phytochemical profile, antioxidant and hepatoprotective potentials of this species. Litsea species also contain structurally diverse and biologically active phytochemicals with broad-spectral biological activities (Agarwal et al., 2011). Therefore, considering this aspect, we investigated on this less explored species of Litsea.

2. Materials and Methods

2.1. Collection of the Plant Material

Litsea floribunda (Bl.) Gamble was collected in the month of May 2012 during the early rainy season from the forests of the Western Ghats (012o17"to 012o 27"N and 075o26"to 075o33"E), Kodagu District, Karnataka, India and identified based on taxonomical parameters. A herbarium specimen of the species is deposited in the herbarium collection of the Department of Studies in Botany, University of Mysore, Manasagangotri, Mysore, India. Plant parts like healthy leaves and stem bark were collected in zip lock polyethylene bags and brought to the laboratory.

2.2. Sample Processing and Extract Preparation

The collected plant parts were washed with water to remove dust and then rinsed with distilled water. Later, they were dried under shade and then in a hot air oven at 40^{o}C overnight until brittle and powdered. The powdered samples were stored in airtight polyethylene bags until use. Fifty grams of dried leaf and bark powder were extracted with solvents in the order of polarity (Hexane > chloroform > ethyl acetate > ethanol > methanol in a Soxhlet apparatus. The liquid obtained after solvent extraction was subjected to drying using a rotary flash evaporator (Superfit Model PBU-6D, India). The residue obtained after flash evaporation of solvents was designated as the dry extracts (Akshatha et al., 2015). The extracts were stored in pre-weighed glass vials and labeled. The aqueous extracts of leaf and stem bark were prepared according to the procedure of Hebbar et al. (2015) by stirring 500 g of the materials in distilled water and boiling for an hour. The extract was filtered using a double layer cheese cloth and the filtrate was evaporated to dryness in a temperature controlled water bath for 72 h (Fisher Scientific, Mumbai, India). The dried powder was scraped, quantified and designated as the dry aqueous extract and used throughout the studies.

2.3. Phytochemical Screening

Qualitative phytochemical screening of solvent extracts was conducted employing standard methods (Harborne, 1973). One gram each of the leaf and bark extracts were dissolved in one ml of respective solvents and tested for the presence of phytochemicals like tannins (Braymer's/Ferric chloride test), flavonoids (Ferric chloride and ammonia test), alkaloids (Mayer's, Wagner's and Dragendrof's tests), saponins (Foam test), cardiac glycosides (Keller-Killani test), terpenoids (Salkowski's test), anthraquinones (Borntrager's test), phlobatannins (Precipitate test), and reducing sugars (Benedict's test).

2.4. Determination of Total Phenolic Content

The total phenolic content in the extracts was estimated by the Folin-Ciocalteau (FC) method employing Gallic acid as standard (mg/mL) (Volluri et al., 2011). The plant extracts were taken in different concentrations (50-250 µg) and were made up to 1000 µL using phosphate buffered saline (PBS, 20 mM, pH-7.4). One ml of FC reagent (1:1 dilution) was added to the test tubes and kept for 3-4 minutes. Later 2.0 mL of Na_2CO_3 (10%, w/v) was added and the mixture was allowed to stand for 45 minutes under dark conditions for incubation. After the specified period of incubation, the absorbance of the samples was measured at 765 nm using UV-Vis spectrophotometer (T60, TTL Technologies,

Bangalore, India). The concentration of total phenolics was expressed in terms of Gallic acid equivalence (µg/g GAE, calculated as mean value ±SD (n=3). The values of the test samples were plotted using the standard curve. All the tests were carried out in triplicates.

2.5. Determination of Antioxidant Activity

The antioxidant activity was evaluated by the following methods:

2.5.1. Radical Scavenging by 1, 1-diphenyl-2-Picryl Hydrazyl (DPPH)

The radical scavenging activity of plant extracts was determined by DPPH (1,1-diphenyl-2-picrylhydrazyl) assay (Pannangpetch et al., 2007). Plant extracts (20-100 µg) were made up to 250 µL using distilled water. Two mL of DPPH solution (0.1 mM) in methanol was added to the extracts and mixed. Blank sample was prepared with one ml of methanol and one ml of DPPH. All the test tubes were kept for incubation in the dark for 20 minutes and the absorbance was measured at 517 nm spectrophotometrically. L-ascorbic acid (5-25 µg) was used as the reference standard and the experiment was carried out in triplicates. The percent scavenging activity was calculated as follows:

$$\%\ \text{radical scavenging} = \frac{\text{Absorbance of the control - Absorbance of the sample}}{\text{Absorbance of the control}} \times 100$$

The concentration of the sample required to scavenge 50% DPPH free radical (IC50) was calculated from the plotting of inhibition (%) against the concentration of extracts.

2.5.2. Ferrous Reducing Antioxidant Power Assay (FRAP assay)

The antioxidant power of the extracts was determined by Ferrous reducing antioxidant power assay (Benzie and Strain, 1996). The FRAP reagent was freshly prepared by adding 10 mM of Tripyridyltriazine (TPTZ) dissolved in 40 mM of HCl, 20 mM of $FeCl_3$ in H2O and 300 mM of acetate buffer (pH 3.6). The fresh working solution was prepared by mixing 25 mL of acetate buffer, 2.5 mL of TPTZ and 2.5 mL of FeCl3. 6H2O, with temperature rise to 37°C. The plant extracts (20-100 µg) were made up to 100 µL by adding methanol to which 3.0 mL of working solution was added and kept at 30°C for 4 minutes. Blank samples were prepared with methanol and a working solution. The absorbance of samples was taken against blank at 593 nm spectrophotometrically. The experiments were performed in triplicates. $FeSO_4$ (5-25 µg) was used as standard for calibration curve and the results were compared with that of standard and expressed in µM Fe (II) /g dry weight.

2.5.3. Reducing Power Assay

Reducing power of the solvent extracts was measured using standard procedure (Oyaizu, 1986). Various concentrations of plant extracts (25-125 µg) were made up to 500 µL by adding PBS. 2.5 mL of Potassium ferricyanide [$K_3Fe(CN)_6$] was added and the mixture was incubated for 20 minutes at 50° C. Later, 500 µL of Trichloroacetic acid (10%, w/v) was added and the mixture was centrifuged at 2500 rpm for 10 minutes. 2.5 mL of supernatant was taken to which equal amount of distilled water and 300 µL of $FeCl_3$ (0.01% w/v) was carefully added. The absorbance of the colored samples was recorded at 700 nm against a blank using spectrophotometer. Blank was prepared using all the reagents without the addition of plant extracts. The experiments were carried out in triplicates. An increase in the absorbance value of the reaction mixture indicates the increased reducing power of the extracts. The average data with standard deviation were recorded for each test sample and represented.

2.6. Hepatoprotective Activity

2.6.1. Animals

Adult albino rats of either sex weighing 140-180 g were selected, housed in the animal house of Sarada Vilas College of Pharmacy, Mysore. The animals were maintained at a temperature of 23±2o C, relative humidity 55±2% and light and dark cycles of 12 L: 12D. They were provided with standardized pellet feed and drinking water ad libitum. The experimental protocol was approved by the Institutional Animal Ethics Committee (IAEC) Reg. No. 706/CPCSEA dt. 1.10.2002. All the experimental procedures were carried out in accordance with the guidelines of CPCSEA.

2.6.2. Chemicals

The drug Silymarin (500 mg) was purchased from Sigma Aldrich Chemical Co. (St. Louis, USA). All other chemicals used were of analytical grade.

2.6.3. Acute Toxicity Studies

Healthy albino rats of either sex were chosen and divided into 4 groups (n=5 in each group). They were fasted overnight and administered with ethanolic and aqueous extracts of L. floribunda orally in a single increasing dose of 1000 mg/kg b.w., 1500 mg/kg b.w., 2000 mg/kg b.w. and 2500 mg/kg b.w of the rats, respectively. The rats were observed continuously for 2, 4 hours and finally for overnight mortality.

2.6.4. Hepatoprotective Study Design

The present study design is as follows:

Group I: Normal was given only vehicle (Guar gum) for 10 days.

Group II: Drug control was administered with standard drug Silymarin p.o. (100 mg/kg b.w.) for 10 days plus paracetamol on the 11th day

Group III: Toxic control was given paracetamol (2 mg/kg b.w.) single dose p.o. on the 11th day.

Group IV & V were administered leaf absolute ethanol extract p.o., 250 & 500 mg/kg b.w., respectively, for 10 days and paracetamol (2 mg/kg b.w., single dose) on the 11th day.

Group VI & VII were administered stem bark absolute ethanol extract *p.o.*250 & 500 mg/kg b.w., respectively, for 10 days and paracetamol (2 mg/kg b.w., single dose) on the 11th day.

Group VIII & IX were administered leaf aqueous extract *p.o.*, 250 & 500 mg/kg b.w., respectively, for 10 days and paracetamol (2 mg/kg b.w., single dose) on the 11th day.

Group X & XI were administered stem bark aqueous extract p.o., 250 & 500 mg/kg b.w.) for 10 days and paracetamol (2 mg/kg b.w., single dose) on the 11th day. After 11 days of treatment, rats of all the groups were anesthetized by diethyl ether 48 hours post-administration. The blood was collected from the retro-orbital plexus. Rats were sacrificed and the liver was carefully dissected, cleaned for extraneous tissue and a portion of it was fixed in Cornoy's fluid (Absolute alcohol: chloroform, 3:1) for histopathological studies. The blood samples thus collected were immediately centrifuged at 2200 rpm for 15 min. The separated serum was analyzed for marker enzymes such as SGOT, SGPT, serum bilirubin, total protein levels (Prism Diagnostics Pvt., Ltd, Thane, India), and ALP (Spinreact, SA, Spain).

2.6.5. Histopathological Studies

Hepatoprotective activity was confirmed through histopathological studies on the liver of rats of all groups. The animals were sacrificed under light anesthesia after 24 hours of last dosage. The livers were dissected out, washed with normal saline and weight determined. Liver tissue was fixed in Cornoy's fluid (Absolute alcohol: chloroform, 3:1) for histopathological studies (Yadav and Dixit, 2003). Sections of all groups were observed under 10X and 40 X of Research microscopes (LabomedOptix, 40 X magnifications) and photographed.

2.7. Statistical Analysis

The results obtained were subjected to statistical analysis using SPSS program (version16.0). The results obtained were compared with the control group in antioxidant assays. The biochemical parameters or the marker enzymes were statistically analyzed using one way analysis of variance (ANOVA) followed by Duncan's Multiple Range Test ($P<0.05$) considered as statistically significant. The data were expressed as mean SEM (n=3 for the antioxidant assays whereas n=5 in hepatoprotectivity study).

3. Results

3.1. Phytochemical Screening

The phytochemical analysis of *L. floribunda* carried out for the various solvent extracts of leaf and stem bark indicated that both the extracts contained saponins, tannins, terpenoids, flavonoids, glycosides and reducing sugars in common (Table 1).

Table 1. Phytochemical screening of leaf and stem bark extracts of *L. floribunda*

Solvent extracts												
	Hexane		Chloroform		Ethylacetate		Absoluteethanol		Methanol		Aqueous	
p	L	B	L	B	L	B	L	B	L	B	L	B
Saponins	+	-	-	-	-	+	+	+	-	+	+	-
Tannins	-	-	-	-	+	-	-	-	-	-	+	+
Alkaloids	-	-	-	-	-	-	-	-	-	-	-	-
Terpenoids	+	+	-	+	+	-	-	-	-	+	-	-
Cardiac glycosides	+	-	+	-	+	+	+	-	+	+	-	-
Flavonoids	-	+	+	+	+	-	-	+	-	+	-	+
Phlobatannins	-	-	-	-	-	-	-	-	-	-	-	-
Anthraquinones	-	-	-	-	-	-	-	-	-	-	-	-
Reducing sugars	-	-	+	-	-	-	-	-	-	-	+	+

(+) = Positive result of the test, (-) = negative result of the test

3.2. Total Phenolic Content

All solvent extracts of *L. floribunda* contained phenolics in various quantities (Table 2). In the leaves, the hexane extract depicted very low content (2.6±0.05 mg/g GAE), while the absolute ethanol extract contained high values (114.8±0.2 mg /g GAE)) and even the stem bark extracts contained phenolics in the same range i.e., 3.5±0.1 mg /g GAE to 117.4±0.1 mg /g GAE.

3.3. Antioxidant Assays

3.3.1. Radical Scavenging by 1, 1-Diphenyl-2-Picryl Hydrazyl (DPPH) Assay

The extracts of *L. floribunda* had the potential to scavenge the DPPH radical, as evident from the IC_{50} values of the leaf and stem bark solvent extracts (Table 2). The aqueous and the hexane extract of leaves possessed IC_{50} values in the range of 29 ±0.1 µg/mL to 216±3.0 µg/mL, respectively. Similarly, in the bark extracts, IC_{50} values of 22 ±0.08 µg/mL was observed for the ethanol extract, while, 240± 1.5 µg/mL was recorded for the hexane extract. Lower IC_{50} value indicates higher activity and visa –versa. The results were compared to the scavenging activity of the standard ascorbic acid ($IC_{50 =}$ 15 µg/mL).

3.3.2. Ferrous Reducing Antioxidant Power Assay (FRAP Assay)

The reducing power of *L. floribunda* extracts were measured and the values are represented (Table 2). The chloroform extracts of both leaf and stem bark exhibited lower values for FRAP assay (1.4±0.2 & 3.3±0.4 µM Fe (II) /g), while high values of 503.9 ±1.05 & 532.2±1.5 µM Fe (II) /g

were observed for the stem bark chloroform and aqueous extracts, respectively.

Table 2. Total phenolic content and antioxidant assay values of solvent extracts of leaf and stem bark of L. floribunda.

0	TPC (mg GAE/ g)	IC_{50} (µg/ml)	FRAP (µM Fe (II)/g)	Reducing power (Absorbance)*
Leaf extracts				
Chloroform	2.6 ± 0.05	241.7±4.3	1.4±0.2	0.38±.01
Ethyl acetate	70.2±1.0	44.2±0.6	150.9±1.3	0.6±.09
Absolute ethanol	114.8±0.2	29.0±0.2	179.4±1.41	2.1±.02
Methanol	82.7±1.0	48.5±0.8	495.7±0.6	1.8±.06
Aqueous	98.9±0.7	25.3±0.4	532.2±1.5	2.9±.09
Stem Bark extracts				
Chloroform	3.5±0.1	154.3±1.0	3.3±0.4	0.4±0.07
Ethyl acetate	96.9±0.1	19.9±0.1	331.5 ±0.5	1.1±0.04
Absolute ethanol	117.4±0.1	21.5±0.8	320.7±0.4	3.2±0.02
Methanol	71.9±0.1	37.3±0.8	309.2±0.9	2.5±0.07
Aqueous	112.4±0.74	24.2±0.2	503.9±1.0	2.1±0.02

All values are expressed as mean ± standard error mean (SEM) (n=3)
*Absorbance represented as two-fold dilutions of the extracts.

3.3.3. Reducing Power Assay

The extracts showed dose-dependent reducing power activity. The reducing power results showed high readings in the ethanol and aqueous extracts of leaf and stem bark. In the present study, high reducing power was noted in the aqueous extract of leaf (2.9 ±0.09), and the absolute ethanol extract of stem bark (3.2±0.02). The results represented in Table 2 indicate that there was an increase in the reducing power of plant extracts as the extract concentration increased.

3.4. Hepatoprotective Studies

Acute toxicity studies conducted to the animal groups were observed continuously for two hours and then occasionally for 4 hours and finally for overnight mortality. The dose up to 2500 mg/kg b.w., was well tolerated without producing any alteration in gross behavioral signs of toxicity and mortality. Based on the observations, the dose selected for the study was fixed at 10% of the maximum tolerated dose that is 250 mg/kg p.o. A higher dose was selected at 500 mg/kg b.w., for administration to the rats.

In the present study, there was a significant increase in the serum levels of SGOT, SGPT, ALP, bilirubin and total proteins in rats treated with paracetamol (G III) as compared to control indicating paracetamol-induced hepatotoxicity (Table 3). Treatment of rats with standard drug Silymarin (G II), and paracetamol on the last day showed decreased levels of marker enzymes ($P < 0.05$) in comparison to paracetamol-treated rats (GIII).

Treatment of rats with the aqueous extract of leaf of *L. floribunda* (250 mg/kg and 500g/kg b.w.,) did not alter the enzyme levels as compared to paracetamol-treated rats. Rats treated with the absolute ethanol extract of leaf (250 mg/kg and 500 mg/kg b.w.) and stem bark (250 mg/kg) showed slight reduction in the serum enzyme levels. The groups treated with the absolute ethanol extract of stem bark (VII;500 mg/kg) and the aqueous stem bark extract (both G X & XI; 250 mg/kg b.w.and 500 mg/kg b.w.) showed significant decrease in the enzyme levels almost close to rats treated with standard drug Silymarin ($P< 0.05$). The liver weight of the toxic group showed an increase (10.68±0.3g) from that of normal controls (5.91±0.2g). Treatment of rats with the absolute ethanol extract of leaf (250 mg/kg and 500 mg/kg b.w.) did not show much decrease in the mean liver weight, but rats treated with the aqueous extracts of stem bark (250 mg/kg and 500 mg/kg b.w.) showed significant decrease in the mean liver weight on par with the rats treated with standard drug Silymarin.

Table 3. Effect of extracts of *L. floribunda* on serum biochemical parameters in paracetamol-induced liver damage in rats

Treatment	Biochemical parameters					
	SGOT (U/L)	SGPT (U/L)	ALP (U/L)	Total Bilirubin (mg/dL)	Total Protein (mg/dL)	Liver weight(g)
GI- Normal	85.8±1.15	67.8±2.8	99.8±2.1	0.67±0.03	6.42±0.1	5.91±0.2
GII-Silymarin (100 mg/kg)	115± 1.2**	84.6±2.2**	117.8±3.0**	0.89±0.22**	7.46±0.8**	6.88±0.4**
GIII-Paracetamol	181.1± 2.4*	166.8±1.8*	255.4±2.2*	2.81±0.17*	4.46±0.4*	10.68±0.3*
G IV-LE 250	163.2 ±1.4	158.4±1.5	239.4±3.0	2.10±0.12	5.90±0.2	8.99±0.45
G V-LE 500	162.4 ±1.7	151.1±0.8	232.3±3.2	1.96±0.08	5.50±0.08	8.81±0.38
G VIII-LA 250	152.1± 1.4	138.8±1.1	184.6±1.7	1.79±0.03	5.22±0.07	8.73±0.27
G IX- LA 500	154.3.±1.6	125.4±2.4	157.4±2.9	1.66±0.06	5.13±0.14	8.51±0.31
G VI- BE 250	149.4 ±1.7	127.4±2.4	170.1±2.5	2.02±0.03	5.80±0.10	8.13±0.27
G VII-BE 500	147.6± 1.2	125.8±2.4	161.1±2.9	1.83±0.02	6.22±0.13**	6.95±0.38**
G X- BA 250	131.1± 2.0**	116.8±1.6**	121.6±2.5**	1.39±0.01	6.04±0.07**	6.31±0.21**
G XI- BA 500	124.2±4.0**	102.8±1.8**	127.4±1.4**	1.37±0.01	6.73±0.08**	6.04±0.34**

LE= leaf ethanol extract, LA= leaf aqueous extract, BE= stem bark ethanol extract, BA= stem bark aqueous extract); Values represent the mean ± SEM; (n=5). 250 & 500 represent dosage of extracts (mg/kg); U/L=Unit/liter; mg=milligram; dL=deciliter;

g=grams; G=grouping of animals; *P<0.05 is considered significant when compared with GI; **P<0.05 is considered significant when compared by GII by Duncan's Multiple Range Test.

3.5. Histopathological Studies

The liver samples of Group I (normal) rats showed normal liver with lobules and hepatocytes (Figure 1A). Group III constituting the toxic group showed distortion of hepatic architecture with foci of lymphocytes intervening the sinusoids which suggested the toxic effects (Figure 1C).

Group II (drug induced) showed normal hepatic morphology with hepatic lobules and hepatocytes (Figure 1B). Group IV, V and VI showed predominantly normal morphology with occasional areas showing lymphatic infiltration and mild distortion of lobular architecture suggesting reduced hepatic toxicity (Figures 1F, J & K). Group VII, VIII and IX rats showed considerable decrease in toxicity effects with moderately dilated hepatic vein and degenerated peripheral hepatocytes infiltration suggesting reduced toxicity (Figures 1G, H & I). Group X, XI showed severe distortion of lobules with totally misplaced portal vein and central vein suggesting toxic changes and less effect of the extracts (Figures 1D & E).

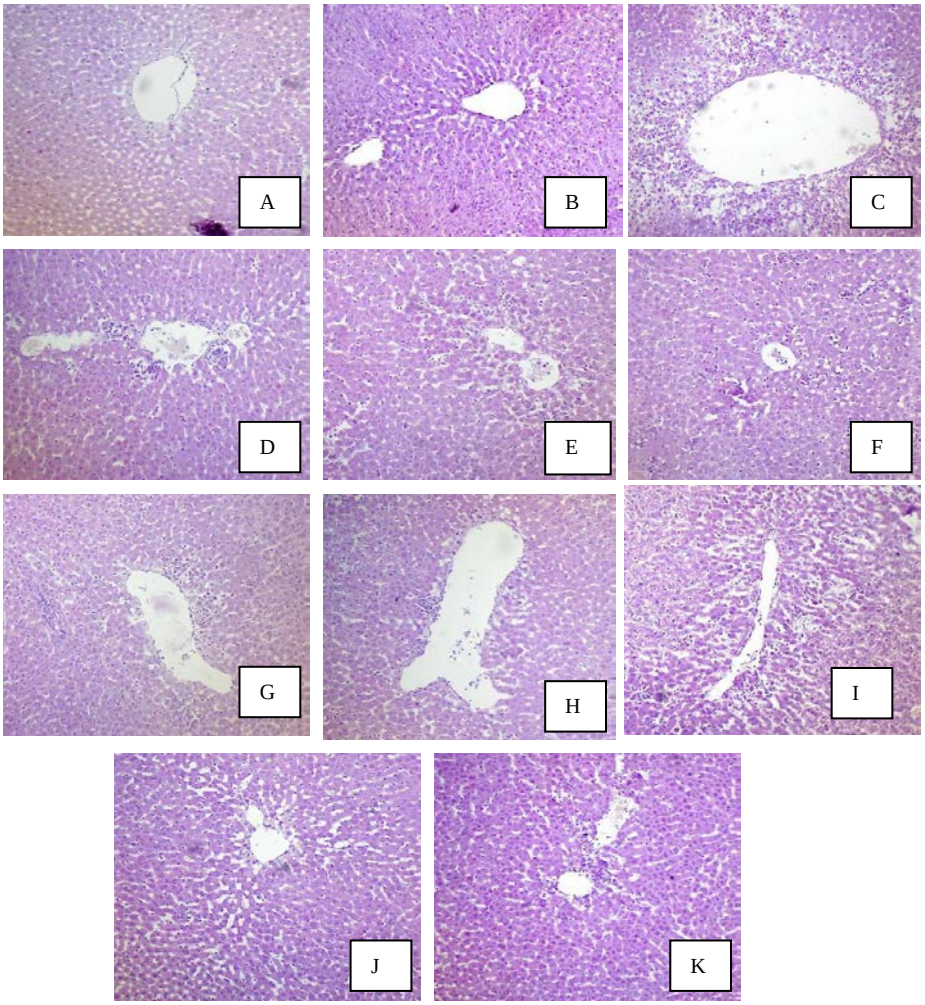


Figure 1. Histopathology of the sections of livers in the experimental groups of animals

A- GI (Normal); B- GII (Standard); C- GIII (Toxic); - D- GIV- LE250; E-GV- LE500; F- VI-BE250; G-GVII- BE500; H-GVIII LA250; I-GIX LA500; J-X BA250; K-XIBA500 BA: Stem Bark aqueous extract; BE: Stem Bark ethanol extract; LA: leaf aqueous extract; LE: leaf ethanol extract; 250 and 500= dosage of extracts (mg/kg b.w.) administered to rats.

4. Discussion

In the present investigation, an endemic *Litsea* species of the Western Ghats was considered for the screening of phytochemicals, antioxidative and hepatoprotective potentials. The Western Ghats are a long coastal hill chain extending from 8° N to 21° N latitude along the west coast of Peninsular India, and is a very narrow hill range stretching between 73°E and 77°E longitude. It covers an area of 180,000 square kilometers and comprises the major portion of the Western Ghats and Sri Lanka Hotspot, one of 34 global biodiversity hotspots for conservation and one of the two in the Indian subcontinent. This area is extraordinarily rich in biodiversity (Bawa *et al*., 2007).

The phytochemicals are the secondary metabolic products produced by plants for their own defense and also bearing potential benefits for humans with antioxidant, antimicrobial, antidepressant, muscle relaxants and anti-inflammatory and many other activities (Briskin, 2000). The presence of flavonoids as major phytoconstituents may be responsible for the antioxidant activity of the extracts. In recent years, due to ethnomedicinal applications, *Litsea* species are being investigated for their pharmacological benefits (Ruth, 2004; Kong *et al*., 2015). Phenolic compounds such as flavonoids, phenolic acids and tannins contribute to the antioxidant capacity of plants and possess biological activities such as anti-inflammatory and anti-carcinogenic activities (Arfan and Kader, 2006; Jia *et al*., 2013). Correlation between the amount of total phenolic content and antioxidant capacity has been established (Li *et al*., 2006). The presence of high phenolic content in the ethanol and aqueous extracts of both leaf and stem bark of *L. floribunda*, may be responsible for the free radical scavenging activity of the extracts.

The presence of flavonoids, reducing sugars and tannins in the bark extract of *L. glutinosa* was responsible for antioxidant activity as the extract showed notable DPPH and H_2O_2 radical scavenging activity (Ruth, 2004). The IC_{50} values of the aqueous leaf (29 .04 ± 0.12µg/mL) and ethanolic bark (21.59 ±0.082 µg/mL) extracts in the present study are closer to the scavenging activity of standard ascorbic acid with IC_{50} value of 14.97 µg/mL. The IC_{50} values obtained for the DPPH assay indeed suggests that the extracts have good antioxidant activity and they could be an important source of plant antioxidants and are comparable to the results obtained in *L. glutinosa*, a species of greater pharmacological interest (Devi and Meera, 2010). On the other hand, the leaf aqueous extract of this species exhibited a lower scavenging activity with IC_{50} value of 30.24 µg/mL.

The Ferrous reducing antioxidant power assay (FRAP assay) is considered as a novel method for assessing antioxidant power (Rabeta and Faraniza, 2013). Higher FRAP values give higher antioxidant capacity as it is based on reducing ferrous ion when antioxidants are the reducing agents. High reducing power of aqueous leaf (532±1.5 µM Fe (II) /g) and stem bark (503.95 ±1.05 µM Fe (II) /g) extracts could be due to the capacity of the extracts to reduce ferrous ion (Fe III) to ferric ion (Fe II) at low pH to form an intense blue colored ferrous tripyridyltriazine (Fe II-TPTZ) complex. The results thus suggest that it has powerful antioxidant activity.

The reducing capacity of extracts is another significant indicator of antioxidant activity. The reducing properties are normally associated with the presence of reductones which are responsible to exert antioxidant activities by breaking the free radical chain by donating a hydrogen atom (Lu *et al*., 2014). The reducing power of *Litsea* extracts is high due to the presence of polyphenols causing reduction of ferric ion (Devi and Meera, 2010). High reducing power (3.2±0.02) of the aqueous leaf and stem bark absolute ethanol extracts (2.9 ±0.09) in *L. floribunda* indicate that the tannins and flavonoids detected in these extracts are responsible for the antioxidant activity.

Hepatotoxicity is an acute adverse effect of drugs in the liver. Several models are available to study and interpret hepatotoxicity levels of which, paracetamol is a widely employed analgesic and antipyretic agent. More consumption of paracetamol results in saturation of these pathways and formation of toxic metabolite (Shenoy *et al*., 2012). The paracetamol induced hepatotoxicity is due to its toxic metabolite, N-acetyl-p-benzoquinoneamine (NAPQ1) which is normally detoxified by glutathione. In paracetamol toxicity, overload of NAPQ1 causes oxidative stress and binds covalently to liver proteins and other macro molecules resulting in hepatic necrosis and hepatic damage due to cease in detoxification (Pandey *et al*., 2012).

In the present study, the aqueous and absolute ethanol extracts of stem bark and leaves of *L. floribunda*, depicting potent IC_{50} values in the DPPH assay was selected for their hepatoprotective potentials. Two dosages of the extracts were administered (250 mg/kg and 500 mg/kg b.w.) on the basis of the results of the acute toxicity studies. The extent of hepatic damage is assessed by histopathological evaluation and the levels of various biochemical parameters like liver marker enzymes in serum. In hepatotoxicity, loss of integrity of hepatocyte membrane leads to cell damage. Therefore, liver marker enzymes present in the cytosol leak out, enter into serum and show an increase in the levels of marker enzymes i.e. SGOT, SGPT, ALP, and bilirubin and decreased level of total protein and albumin in serum (Chaudhari *et al*., 2009). The animal groups treated with the absolute ethanol (VII:500 mg/kg) and the aqueous (X: 250 mg/kg and XII: 500 mg/kg) stem bark extracts showed significant decrease in the enzyme levels almost close to rats treated with standard drug Silymarin ($P< 0.05$).

Hepatic damage induced by paracetamol resulted in elevated levels of SGOT, SGPT, ALP and bilirubin reflects the liver damage and indicates a loss of functional integrity of cell membranes in the liver which is further reflected in the histopathological studies. Synthesis of protein is one of the most important liver functions. Liver damage causes disruption and disassociation of polyribosomes on endoplasmic reticulum and thereby reducing the biosynthesis of protein. Restoring the normal levels of protein is an important parameter for liver recovery (Navarro and Senior, 2006). Treatment with paracetamol resulted in a decrease in liver protein levels. The significant reduction in the level of marker enzymes and total protein in the serum due to the administration of stem bark aqueous and absolute ethanol extracts of *L. floribunda* treated groups provides key evidence for the hepatoprotectivity of the extracts. So far, hepatoprotective potentials have been described and documented for one species, *L. coreana* var. *lanuginose*, commonly known as hawk tea, tested in the carbon tetrachloride-induced hepatotoxicity model (Zhao, 2013). Therefore, owing to the observed antioxidant and hepatoprotective potentialities of the various extracts tested in the present study, *L. floribunda* and its phytochemicals may offer potential therapeutic benefits.

5. Conclusion

Litsea floribunda is an important endemic species of the Western Ghats of southern India. An attempt has been made in the present study to investigate the phytochemicals, antioxidative and hepatoprotective potentials of the solvent extracts. The obtained results justify the fact that the absolute ethanol and aqueous extracts of both leaf and stem bark with high phenolic content exhibited higher antioxidant activity than other solvent extracts. These extracts contain flavonoids as well as other phenolic compounds that may be responsible for their antioxidant activities. The aqueous and absolute ethanol stem bark extracts in both lower and higher doses have shown potent antioxidant activity which is confirmed through histopathological studies. Further, studies directed towards the fractionation of extracts and identification of compounds responsible for the antioxidant and hepatoprotectivity are deemed necessary future work.

Conflict of interest

The authors declare no conflict of interest in the publication of the manuscript.

Acknowledgement

The authors are thankful to the Chairman, DOS in Botany, University of Mysore, Manasagangotri, India for providing the necessary research facilities. The present study is assisted by the financial assistance from the University Grants Commission (UGC) Minor Research Grant with the approval letter no. No. F. MRP/ 12th Plan/14-15/KAMY007 Dt. 10-12-2014 to the first author.

References

Agarwal N, Chowdhary AS, Sharma MC and Dobhal MB. 2011.Chemical constituents of plants from the genus *Litsea. Chem Biodivers*, **8**: 223-243.

Akshatha JV, Prakash, HS and Nalini MS. 2015. Antioxidative and α-amylase inhibitory potentials of medicinal plants from the Western Ghats of southern India. *Pharm Lett*, **7**: 10-17.

Alqasoumi SI. 2014. Valuation of hepatoprotective and nephroprotective activities of *Scrophularia hypericifolia* growing in Saudi Arabia. *Saudi Pharm J*, **22**: 258-263.

Arfan M and Kader A. 2006. Antioxidant properties-tannins from *Litsea monopetala*. *Polish J Food and Nutr Sci*, **34**: 66-69.

Aseervatham GSB, Sivasudha T, Sasikumar JM, Christabel PH, Jeyadevi R, and Ananth DA. 2013. Antioxidant and hepatoprotective potential of *Poutera campechiana* on acetaminophen-induced hepatic toxicity in rats. *J Physiol Biochem*, DOI.10.1007/s13105-013-0274-3.

Bawa KS, Das A, Krishnaswamy J, Karanth KU, Kumar, NS and Rao, M. 2007. Ecosystem profile: Western Ghats and Sri Lanka biodiversity hotspot,Western Ghats region. *Critical Ecosystem Partnership Fund Report*. pp. 1-3.

Benzie IF and Strain JJ. 1996. The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": the FRAP assay. *Analyt Biochem*, **239**: 70-76.

Bhagwat SA, Kushalappa CG, Williams PH and Brown ND. 2005. The role of informal protected areas in maintaining biodiversity in the Western Ghats of India. *Ecology & Soc*, **10**: 8. Online URL: http://www.ecologyandsociety.org/vol10/iss1/art8/.

Bhuniya T, Singh P, and Mukherjee SK. 2010a. An account of the species of *Litsea* LAM. (Lauraceae) Endemic to India. *Bangl J Plant Taxon*, **17:** 183-191.

Bhuniya T, Singh P, and Mukherjee SK. 2010b. *Litsea cubeba*-medicinal values-brief summary. *J Trop Med Plants*, **11**: 124-130.

Briskin DP. 2000. Medicinal plants and phytomedicines, linking plant biochemistry and physiology to human health. *Plant Physiol*, **124**: 507-514.

Chaudhari NB, Chittan KP, and Patil VR. 2009. Hepatoprotective activity of *Cassia Fistula* seeds against paracetamol-induced hepatic injury in rats. *Arch Pharm Sci Res*, **1**: 218-221.

Devi P, and Meera R, 2010. Study of antioxidant, anti-inflammatory and wound healing activity of extracts of *Litsea glutinosa. J Pharma Sci & Res*, **2**: 155-163.

Koche D, Shirsat R, Imran S, and Bhandange DG. 2010. Phytochemical screening of eight traditionally used ethno medicinal plants from Akola district (MS) India. *Int J Pharm Bio Sci*, **1**:2 53 – 256.

Girish KE, Pradeep KG, Sasikala K and Shivadasan KK. 2014. Plants used in traditional herbal shampoos (thaali) of Kerala, India: a documentation. *Asia Pacific J Res*, **10**: 56-63.

Harborne JB. 1973. **Phytochemical Methods**. Chapman and Hall Ltd., London., pp. 49-188.

Hebbar DR, Savitha G and Nalini MS. 2015. Aqueous leaf extracts of *Schefflera venulosa* and *S. wallichiana* protects the liver against Carbon tetrachloride (CCl_4) - induced hepatic damage in albino rats. *Am J Pharm Tech Res*, **5**: 328-340.

Jia X, Dong L, Yang Y, Yuan S, Zhang Z and Yuan M. 2013. Preliminary structural characterization and antioxidant activities of polysaccharides extracted from *Litsea coreana* (Hawk tea). *Carbohydr Polym*, **95**: 195-199.

Kong DG, Zhao Y, Li GH, Chen BJ, Wang XN, Zhou HL, Lou HX, Ren DM and Shen T. 2015. The genus *Litsea* in traditional Chinese medicine: an ethnomedical, phytochemical and pharmacological review. *J. Ethnopharmacol.*, 164: 256-264. doi: 1016/j.jep. 2015.02.020.

Li HB, Cheng KW, Wong CC, Fan KW, Chen F and Jiang Y. 2006. Evaluation of antioxidant capacity and total phenolic content of different fractions of selected microalgae. *Food Chem*, **102**: 771-776.

Lu Y, Shipton FN, Khoo TJ, and Wiart C. 2014. Antioxidant activity determination of *Citronella* and crude extracts of *Cymbopogon citratus* by three different methods. *J. Pharm. Pharmacol*, **5**: 395-400.

Luper S. 1998. A review of plants used in the treatment of liver diseases: Part 1. *Altern Med Rev*,**3**: 410-421.

Navarro VJ, and Senior JR. 2006. Drug-related hepatotoxicity. *N Engl J Med*, **35**: 731–739.

Oyaizu M. 1986. Studies on products of browning reactions: antioxidant activities of products of browning reaction prepared from glucosamine. *J Nutr*, **44**: 307-315.

Pandey N, Barve D, Prajapati N, and Dubey BK. 2012. Evaluation of hepatoprotective activity of ethanolic extract of *Arisaema leschenaultia* Blume tuber in paracetamol-induced hepatotoxicity in Swiss albino mice. *Int J Res Pharma Biomed Sci*, **3**: 312- 318.

Pannangpetch P, Laupattarakasem P, Kukongviriyapan V, Kukongviriyapan U, Kongyingyoes B and Aromdee C. 2007. Antioxidant activity and protective effect against oxidative hemolysis of *Clinacanthus nutans. Sangklanakorin J Sci Technol*, **29**: 1-9.

Rabeta MS, and Faraniza RN. 2013. Total phenolic content and ferric reducing antioxidant power of the leaves and fruits of *Garcinia atroviridis* and *Cynomera cauliflora*. *Int Food Res J*, **20**: 1691-1696.

Ruth B. 2004. Medicinal properties of *Litsea glutinosa* Lour. and *Litsea monopetala*. *e-Planet*, **2**: 94-95.

Sheikh RA, Babu DJM, Venkatrao N, Irene PR, More S, and Turaskar A. 2012. Hepatoprotective activity of alcoholic and aqueous extracts of bark of *Bassia latifolia* Roxb. against paracetamol induced hepatotoxicity in rats. *Pharm Lett*, **4:** 1272-1284.

Shenoy S, Kumar H, Thashma, Nayak V, Prabhu K, Pai P, Warrier I, Somayaji, Madhav V, Bairy KL and Kishore A 2012. Hepatoprotective activity of *Plectranthus ambolonicus* against paracetamol–induced hepatoprotectivity in rats. *Int J Pharmacol Clin Sci*, **1**: 52-58.

Volluri SS, Bammidi SR, Chippada SC and Vangalapati M. 2011. *In vitro* antioxidant activity and estimation of total phenolic content in methanolic extract of *Bacopa monniera*. *Rasayan J Chem*, **4**: 381-386.

Yadav MS, Kumar A, Singh A, Sharma US and Sutar N. 2011. Phytochemical investigation and hepatoprotective activity of *Cissampelos pareira* against CCl_4-induced hepatotoxicity," *Asian J Pharm Health Sci*, **1**: 106-110.

Yadav NP and Dixit VK. 2003. Hepatoprotective activity of leaves of *Kalanchoe pinnata* Pers. *J Ethnopharmacol*, **86**: 197-202.

Zhao X. 2013. Hawk tea (*Litsea coreana* Levl. var. *lanuginose*) attenuates CCl_4-induced hepatic damage in Sprague-Dawley rats. *Exp Ther Med*, **5**: 555-560.

JJBS

Volume 9, Number 3, June .2016
ISSN 1995-6673
Pages 173 - 177

Jordan Journal of Biological Sciences

Catechin Protects Against Dyslipidemia and Nephro-Hepatototoxicity Induced in Rats Exposed to Arsenic

Olusegun K. Afolabi[1,*], Regina N. Ugbaja[2], Abimbola Arinola[1], Olubukola S. Olorunnisola[1], Gbadebo E. Adeleke[1].

[1]*Department of Biochemistry, Ladoke Akintola University of Technology, Ogbomoso, Nigeria*

[2]*Department of Biochemistry, Federal University of Agriculture, Abeokuta, Nigeria*

Received: June 6, 2016 Revised: July 9, 2016 Accepted: July 18, 2016

Abstract

Arsenic poisoning is a major environmental event affecting millions worldwide and its treatment with chelating agents has met with limited success. While arsenic toxicity affects multiple systems in the human body, its mode of action has not been fully elucidated. The present study therefore, investigated the possible protective effects of catechin against hepatorenal damage and dyslipidemia induced by arsenic exposure. Rats were exposed to arsenic (100 ppm) through their drinking water and were treated with catechin (40 mg/kg and 80 mg/kg, body weight) for 30 days. Arsenic exposure resulted in liver dysfunction obvious with increased activities of the hepatic enzymes alanine aminotransferase (ALT), alkaline phosphatase (ALP) and aspartate aminotransferase (AST). This was accompanied with significant elevation of kidney function markers urea and creatinine ($p < 0.05$). Furthermore, arsenic caused the distortion of lipid metabolism resulting in hypercholesterolemia, hypertriglyceridemia and increased plasma phospholipid in the animals. Co-treatment with catechin effectively protected against arsenic-mediated hepatotoxicity, prevented renal damage and restored lipid homeostasis in the rats. The present data indicate the ability of catechin to potentially prevent arsenic-induced nephro-hepatotoxicity and dyslipidemia in rats.

Keywords: Arsenic, Catechin, Hepatotoxic, Nephrotoxic, Dyslipidemia.

1. Introduction

Environmental pollution by heavy metals has become a major global concern, most especially in the developing countries. Arsenic (As) because of its highly toxic nature is one of the most important of these metals (ATSDR, 2007). It is a carcinogen and is considered to be one of the most hazardous chemicals. It is widely distributed in nature and is anthropogenically released into the environment through industrial processes and agricultural usage (Flora et al., 1995; ATSDR, 2007). Studies have shown that arsenic exposure could lead to a variety of biochemical and physiological dysfunctions in humans and experimental animals (Lena et al., 2014). Toxicities induced by arsenic have been associated with different forms of cancer, apoptosis, genetic damage, hematological disorders, and cardiovascular diseases, among many others (Prakash et al., 2014). Environmental exposures to arsenic majorly occur through the consumption of drinking water from contaminated groundwater sources (Murcut, 2012). In some of these groundwater, arsenic concentrations range far above the current maximum permissible limit of 10 µg/l recommended by World Health Organization (WHO, 2011).

Traditionally, the treatment of arsenic poisoning is carried out by administering a chelating agent such as 2, 3-dimercapto-1-propanol (BAL). More orally effective and less toxic dithiol chelating agents, sodium 2,3-dimercaptosuccinic acid (DMSA) and sodium 2,3-dimercaptopropane 1-sulphonate (DMPS), are also in use (Flora et al., 1995; Aposhian and Aposhian., 1989). However, chelation therapy as a form of arsenic poisoning treatment is rife with side effects and is ineffective against some forms of arsenic toxicity (Gupta and Flora, 2005). Oxidative stress has been suggested to be one of the mechanisms by which arsenic effects its toxicity (Kumar et al., 2014). Generally, inorganic arsenic increases reactive oxygen species (ROS) by binding vicinal thiols or sulfur-containing groups (Majhi et al., 2011; Patra et al., 2012). It is, therefore, reasonable to hypothesize that an antioxidant agent could protect against some of the effects induced by arsenic exposure.

In the present study, we test the hypothesis that catechin, a widely distributed flavonoid present in

* Corresponding author. e-mail: okafolabi@lautech.edu.ng.

plant foods and drinks, possessing strong antioxidant activity can protect against arsenic-induced hepatotoxicity, nephrotoxicity and dyslipidemia in rats. The demonstration of this may be an important finding for developing new treatment for arsenic poisoning.

2. Materials and Method

2.1. Chemicals

Sodium arsenite and catechin were obtained from Sigma-Aldrich, Germany. All other chemicals used were of analytical grade.

2.2. Animals and Treatment

Thirty five male Wistar rats (bred in the Animal House, Faculty of Basic Medical Sciences, LAUTECH) weighing an average of 120g were used for the present study. The animals were housed in an animal room with controlled temperature (22±2˚C) and a regular 12h light-dark cycle (06:00-18:00h). They were allowed to acclimatize for 14 days before used for the experiment. They were allowed free access to rat chow and drinking water. The experimental protocol was approved by the Biochemistry Department Ethic Committee on Research of LAUTECH.

Animals were randomly divided into 5 groups of 7 rats each and were treated as below for a period of 30 days: Group I, normal animals; Group II, arsenic (in the form of sodium arsenite), 100 ppm in drinking water; Group III, Catechin at 40 mg/kg body weight/day, orally; Group IV, arsenic (as in Group II) plus Catechin at 40 mg/kg body weight/day, orally; Group V, arsenic (as in Group II) plus Catechin at 80 mg/kg body weight/day, orally.

The doses for arsenic and catechin were selected based on previously published studies (Afolabi et al., 2015; Chopra et al., 2004). At the end of the treatment period, blood was collected from the animals into heparinized tubes by cardiac puncture under light ether anesthesia after an overnight fast. The blood was centrifuged at 3000g for 10 min to separate the plasma. Samples were stored at -20oC for subsequent biochemical analyses.

2.3. Biochemical Analyses

Plasma concentrations of total cholesterol, triglyceride and phospholipids were determined with standard diagnostic kits (Chemelex®, Barcelona, Spain). HDL cholesterol was analyzed with the same diagnostic kit for total cholesterol after very low density lipoproteins (VLDL) and low density lipoproteins (LDL) were precipitated with heparin-MnCl2 solution as described by Gidez et al. (1982). LDL cholesterol was estimated using Friedewald formula (1972). Plasma urea and creatinine were determined spectrophotometrically by the method of Patton and Crouch (1977) and Henry et al.'s method (1974), respectively. The activities of plasma hepatic marker enzymes, alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were determined by the method of Reitman and Frankel (1957), while alkaline phosphatase (ALP) was assayed using the method of Bessey et al. (1946).

3. Results

Figure 1 shows the levels of AST, ALT and ALP in the plasma of rats. In the group exposed to arsenic only, ALT level was significantly increased when compared with the control group (38.16 ± 3.63 and 26.14 ± 2.59 U/L, respectively, $p < 0.05$). Treatment of arsenic exposure with either high or low dose catechin lowered ALT level significantly when compared with arsenic exposure only (19.18 ± 2.13 and 26.64 ± 2.03 UL, respectively, $p < 0.05$). There was no significance difference between control and catechin only groups ($p > 0.05$). Similarly, arsenic caused drastic elevation of both ALP and AST in the rats, compared to the control. Arsenic increased both activities from 26.66 ± 3.79 and 77.20 ± 4.05 to 48.66 ± 3.07 and 93.60 ± 4.84 U/L, respectively. High and low dose catechin however, reversed this trend and significantly lowered ALP to 26.26 ± 1.83 and 33.51 ± 2.83 U/L, and AST to 87.70 ± 2.65 and 70.60 ± 3.69 U/L respectively.

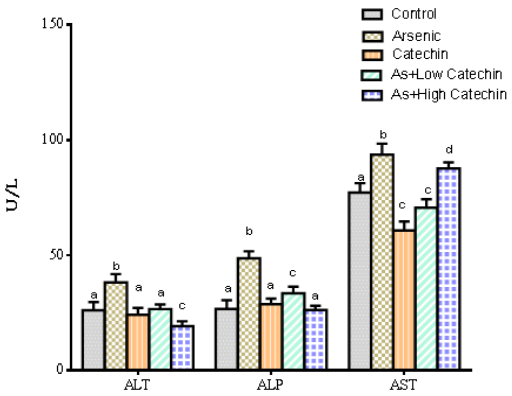


Figure 1. Effects of catechin on the levels of ALT, ALP and AST in the plasma of rats exposed to arsenic. Values are mean ± SD. Bars bearing different alphabets are significantly different from each other at $p < 0.05$.

Arsenic exposure resulted in a marked impairment of kidney function as demonstrated by the high levels of urea and creatinine shown in Figure 2. In arsenic only group, urea level was significantly increased when compared to the control and catechin only groups (45.81 ± 1.24, 18.23 ± 2.04 and 24.67 ± 1.32 mg/dl, respectively, $p < 0.05$). Treatment with either low or high dose catechin reduced high urea level induced by arsenic exposure (32.74 ± 1.58, and 35.41 ± 2.74 mg/dl, respectively, $p < 0.05$). Creatinine was also significantly elevated in arsenic only group compared with control, and catechin only groups (1.82 ± 0.10, 0.94 ± 0.03 and 0.89 ± 0.04 mg/dl, respectively, $p < 0.05$). Creatinine was lowered by treatment with both low and high doses of catechin (1.15 ±0.06 and 1.05 ± 0.05 mg/dl, respectively, $p < 0.05$).

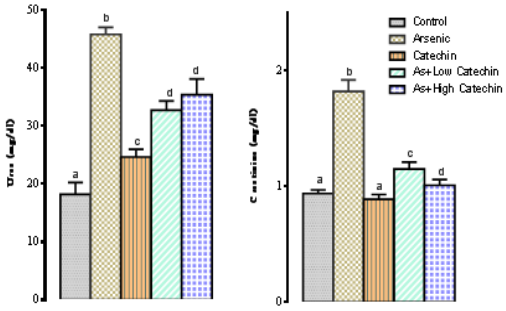


Figure 2. Effects of catechin on plasma urea and creatinine levels in rats exposed to arsenic. Values are mean ± SD. Bars bearing different alphabets are significantly different from each other at $p < 0.05$.

The present study demonstrates that the arsenic exposure induced dyslipidemia in rats. Estimated values of plasma cholesterol, triglyceride and phospholipid levels are depicted in Figure 3. There were no statistically significant changes in plasma cholesterol, triglyceride and phospholipid between control and catechin only groups ($p > 0.05$). However, arsenic exposure caused significant elevation of these lipids compared with the control (90.11 ± 4.59, 48.91 ± 3.81 and 127.36 ± 7.25 mg/dl vs. 50.71 ± 6.01, 40.83 ± 3.75 and 96.69 ± 6.50 mg/dl, respectively, $p < 0.05$). Both high and low dose catechin treatments restored plasma triglyceride and phospholipid concentrations back to control levels ($p > 0.05$). In addition, low and high catechin treatments significantly lowered cholesterol level when compared with arsenic only group (68.09 ± 5.09 and 58.47 ± 3.43 mg/dl, respectively, $p < 0.05$), but the concentrations were not comparable to the basal level.

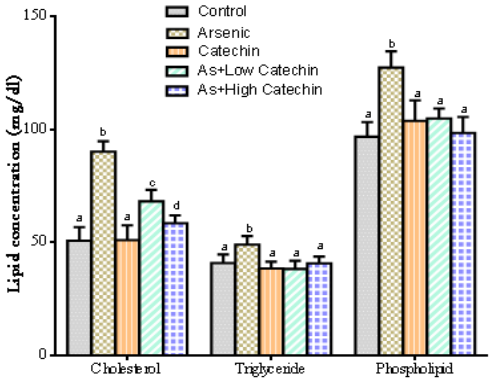


Figure 3. Effects of catechin on the plasma cholesterol, triglyceride and phospholipid concentrations in rats exposed to arsenic. Values are mean ± SD. Bars bearing different alphabets are significantly different from each other at $p < 0.05$.

Arsenic caused the lowering of the HDL concentration while increasing the LDL levels in the rats (Fig. 4). The metal induced a significant 63% reduction in HDL level in rat which was subsequently reversed, to different degrees by catechin treatments. Low dose catechin raised the HDL concentration from 7.89 ± 0.57 mg/dl in arsenic alone group to 25.67 ± 1.96 mg/dl. The high dose treatment also increased the lipoprotein level (14.43 ± 1.07 mg/dl), albeit the increment was not comparable to that induced by the low dose catechin treatment. LDL concentration was significantly elevated by arsenic from 8.67 ± 0.79 in control to 9.78 ± 0.76 mg/dl ($p < 0.05$). Catechin only group had their LDL concentration lowered significantly ($p < 0.05$), compared with the control group (7.67 ± 0.99 mg/dl). The treatment of arsenic group with low and high doses of catechin also brought about significant reduction of LDL levels (7.63 ± 0.86 and 8.12 ± 0.69 mg/dl, respectively, $p < 0.05$).

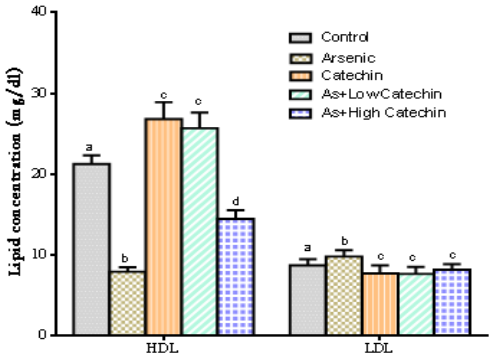


Figure 4. Effects of catechin on HDL and LDL concentrations in rats exposed to arsenic. Values are mean ± SD. Bars bearing different alphabets are significantly different from each other at $p < 0.05$.

4. Discussion

In the present study, it was demonstrated that arsenic exposure could cause dyslipidemia by disrupting lipid homeostasis. Additionally, the findings indicated that arsenic induces liver and kidney injury in rats. However, catechin was able to prevent these toxicities caused by arsenic.

Plasma aminotransferase activities are sensitive indicators of liver damage. This damage may alter transport function and membrane permeability of hepatocytes, resulting in enzymes leakage from the cell. The efflux of these enzymes from the hepatocytes causes a depletion in AST, ALP and ALT levels with concomitant elevation of their activities in the plasma. In the present study, the plasma activities of these enzymes were significantly elevated by arsenic exposure, suggestive of hepatic damage induced by the metalloid. Similar report of liver damage induced by arsenic exposure was given by Miltonprabu and Sumedha (2014). The liver is the major organ involved in xenobiotic metabolism and is thus, susceptible to oxidative damage caused by increased Reactive Oxygen Species (ROS). Arsenic has been demonstrated to generate oxidative stress (Nandi *et al*., 2006), and the involvement of ROS has been linked with arsenic pathogenicity. Experimental studies suggest that lipid peroxidation, one of the determinants of oxidative stress induced by ROS, contributes to arsenic-induced oxidative damage of membrane (Flora *et al*., 2008). Arsenic affinity for –SH group of proteins enables it to conjugate and

form covalent attachment with intracellular GSH and inhibit glutathione reductase activity, in addition to glutathione synthesis (Quig, 1988). This leads to depletion of intracellular thiols, escalating oxidative damage to several biological macromolecules (Hansen *et al*., 2006). The elevated level of the liver enzymes in the plasma may thus be attributed to this process. Catechin treatment on the other hand, was able to reverse the damage induced by arsenic. It was able to restore the enzymes' activities to the basal levels, especially the high dose treatment. In the case of AST, however, treatment with catechin at high dose even though lowered the enzyme level below arsenic-induced activity, was unable to restore it to normal as did the low dose treatment. The ability of catechin to protect against liver injury may be predicated upon its antioxidant activity, which could have prevented the escalation of lipid peroxidation induced by arsenic (Mishra and Flora, 2008; Nandi *et al*., 2006), leading to membrane damage in the organ. Studies have already demonstrated catechin's protective effect against ROS generation, as well as, the ability of polyphenols to induce antioxidant enzymes (Zhang *et al*., 2014; Du *et al*., 2007). Catechin also possesses chelating property and might have chelated with arsenic, removing the metal from circulation thereby, preventing its damaging effects (Sugihara *et al*., 2001).

The protective property of catechin, through its antioxidant ability, against the subsequent membrane damage induced by ROS generation seemed to be at play in its protection of the kidney against arsenic toxicity. Renal dysfunction was demonstrated by arsenic exposure in the present study as exhibited by the increase in plasma urea and creatinine levels. Several studies have demonstrated arsenic toxicity of in the kidney (Eom *et al*., 2011; Chen *et al*., 2011) and the major mechanism of kidney damage by this metalloid has been suggested to be oxidative stress (Jomova *et al*., 2011). The increased generation of ROS by arsenic has been implicated in the stimulation of proinflammatory and profibrogenic cytokines (Brunati *et al*., 2010), which are significant causal factors in nephrotoxicity. Oxidative stress may have stimulated lipid peroxidation, damaging cell membranes and organelles, releasing reactive aldehydes with potent proinflammatory properties (Zhang *et al*., 2014). Catechin as was observed in the liver, markedly reversed the damage induced by arsenic in the kidney. Although a total restoration of renal function was not obtained, plasma urea and creatinine levels in the treatment groups were significantly lowered by catechin, compared with the arsenic intoxicated group.

Although there are differences in the status of individual lipids reported, studies have implied that arsenic exposure can alter lipid metabolism, resulting in cardiovascular disorder (Afolabi *et al*., 2015; Muthumani and Prabu, 2014). In the present study, exposure to this toxicant induced hypercholesterolemia and hypertriglyceridemia in addition to causing an elevation in phospholipid and LDL levels in rats. On the other hand, HDL was drastically reduced in the animals by the metal. Co-treatment of arsenic with catechin however, significantly reduced levels of cholesterol, triglyceride, phospholipids and LDL compared with the group treated with arsenic alone. Arsenic has already been demonstrated to stimulate HMG CoA reductase, the rate limiting enzyme in cholesterol biosynthesis (Afolabi *et al*., 2015), resulting in the elevation of the lipid in the plasma. The reduction in plasma cholesterol concentration could result from the inhibition of intestinal absorption of the lipid as suggested by Ikeda (2005). Alternatively, catechin might have prevented the accumulation of the lipid by stimulating the activity of LCAT and other enzymes involved in cholesterol catabolism.

The hydrolysis of triglycerides leads to an efflux of Free Fatty Acid (FFA) from the adipose tissue into the circulation. Physiological stress, such as induced by arsenic exposure, can increase the mobilization of this lipid from the adipose tissue (Newsholme and Start, 1981) and arsenic exposure has been linked with elevated plasma free fatty acid (FFA) concentration (Afolabi *et al*., 2015). High circulating FFA inhibits the activity *of* lipoprotein lipase (Saxena *et al*., 1989) which can disturb lipid homeostasis with the resultant hypertriglyceridemia, and decreased HDL cholesterol level as observed in this study (Afolabi *et al*., 2015). Catechin's ability to restore the distorted lipid homeostasis could be explained by it having a stabilizing effect on lipoprotein lipase, probably through its free radical scavenging properties, thereby preventing the enzyme's inhibition by the arsenic. Similar action of catechin against diabetes-induced hypertriglyceridemia in rats was reported by Mostafa (2013). Catechin may also possess the ability to inhibit pancreatic lipase, causing a delay in fat absorption that may be responsible for the lowering of the plasma triglyceride concentration observed.

5. Conclusion

On the basis of the results obtained from the present study, it is further demonstrated that arsenic exposure can result in dyslipidemia, nephrotoxicity and hepatotoxicity. But more significantly, the data suggest that the flavonoid, catechin possesses the potential to ameliorate hepatic and renal injury, as well as, modify the disruption to lipid metabolism induced by arsenic exposure through drinking water.

References

Afolabi OK, Wusu AD, Ogunrinola OO, Abam E, Babayemi DO, Dosumu OA, Onunkwor OO, Balogun EA, Odukoya OO and Ademuyiwa O. 2015. Arsenic-induced dyslipidemia in male albino rats: comparison between trivalent and pentavalent inorganic arsenic in drinking water. *BMC Pharmacol Toxicol,* **16**:15.

Agency for Toxic Substances and Disease Registry. 2007. Toxicological Profile for Arsenic. Agency for Toxic Substances and Disease Registry.

Aposhian HV and Aposhian MM. 1989. Newer developments in arsenic toxicity. J Amer Coll Toxicol, 8: 1297–1305.

Bessey OH, Lowry MD and Brock A. 1946. A method for the rapid determination of alkaline phosphatase with 5 mm^3 of serum. *J Biol Chem*, 164:321–329.

Brunati AM, Pagano MA, Bindoli A and Rigobello MP. 2010. Thiol redox systems and protein kinases in hepatic stellate cell regulatory processes. *Free Radic Res*, **44**:363‐ 378.

Chen JW, Chen HY, Li WF, Liou SH, Chen CJ, Wu JH and Wang SL. 2011. The association between total urinary arsenic concentration and renal dysfunction in a community‐ based population from central Taiwan. *Chemosphere*, **84**: 17‐ 24.

Chopra K, Singh D and Chander V. 2004. Nephrotoxicity and its prevention by catechin in ferric nitrilotriacetate promoted oxidative stress in rats. *Hum Exp Toxicol*, **23**:137-143.

Du Y, Guo H and Lou H. 2007. Grape seed polyphenols protect cardiac cells from apoptosis via induction of endogenous antioxidant enzymes. *J. Agric. Food Chem*, **55**: 1695-1701.

Eom SY, Lee YC, Yim DH, Lee CH, Kim YD, Choi BS, Park CH, Yu SD, Kim DS, Park JD and Kim H. 2011. Effects of low‐ level arsenic exposure on urinary N‐ acetyl‐ β‐ D‐ glucosaminidase activity. *Hum Exp Toxicol*, **30**:1885‐ 1891.

Flora SJ, Dube SN, Arora U, Kannan GM, Shukla MK, et al. 1995. Therapeuticpotential of meso 2,3-dimercaptosuccinic acid or 2,3-dimercaptopropane1-sulfonate in chronic arsenic intoxication in rats. *Biometals*, **8**: 111-116.

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

Gidez LT, Miller GH, Burnstein M, Slagle S and Eder HA. 1982. Separation and quantitation of subclasses of human plasma high density lipoproteins by a simple precipitation procedure. J Lipid Res, 23:1206–1223.

Gupta R and Flora SJ. 2005. Protective value of Aloe vera against some toxic effects of arsenic in rats. *Phytother Res*, **19**(1): 23-28.

Henry RJ, Cannon DC and Winkelman W. 1974. **Clinical Chemistry Principals and Techniques**, eleventh ed. Happer and Row Publishers.

Ikeda I, Tsuda K, Suzuki Y, Kobayashi M, Unno T, Tomoyori H, Goto H, Kawata Y, Imaizumi K, Nozawa A and Kakuda T. 2005. Tea catechins with a galloyl moiety suppress postprandial hypertriacylglycerolemia by delaying lymphatic transport of dietary fat in rats. *J Nutr*, **135**:155-159.

Jomova K, Jenisova Z, Feszterova M, Baros S, Liska J, Hudecova D, Rhodes CJ and Valko M. 2011. Arsenic: toxicity, oxidative stress and human disease. *J Appl Toxicol*, **31**:95‐ 107.

Kumar S, Yedjou CG and Tchounwou PB. 2014. Arsenic trioxide induces oxidative stress, DNA damage, and mitochondrial pathway of apoptosis in human leukemia (HL–60) cells. *J Exp Clin Cancer Res*, **33**:1 – 12.

Lena QM, Hong-Jie S, Bala R, Bing W, Jun L and Li-Ping P. 2014. Arsenic and selenium toxicity and their interactive effects in humans. *Environ Inter*, **69**:148–158.

Majhi CR, Khan S, Leo MD, Manimaran A, Sankar P and Sarkr SN. 2011. Effects of acetaminophen on reactive oxygen species and nitric oxide redox signaling in kidney of arsenic-exposed rats. *Food Chem Toxicol*, **49:**974–982.

Miltonprabu S and Sumedhaa NC. 2014. Arsenic-induced hepatic mitochondrial toxicity in rats and its amelioration by diallyl trisulfide. *Toxicol Mech Meth*, **4**:124-135.

Mishra D and Flora SJ. 2008. Differential oxidative stress and DNA damage in rat brain regions and blood following chronic arsenic exposure. *Toxicol Ind Health*, **24**: 247‐ 256.

Mostafa UE. 2013. Effect of Green Tea and Green Tea Rich with Catechin on Blood Glucose Levels, Serum Lipid Profile and Liver and Kidney Functions in Diabetic Rats. *Jordan J Biol Sci*, **7**:7-12.

Murcott S. 2012. **Arsenic contamination in the world: An international sourcebook**. IWA Publishing, London, UK.

Muthumani M and Prabu SM. 2014. Silibinin potentially attenuates arsenic-induced oxidative stress mediated cardiotoxicity and dyslipidemia in rats. *Cardiovasc Toxicol*, **14**:83–97.

Nandi D, Patra RC and Swarup D. 2006. Oxidative stress indices and plasma biochemical parameters during oral exposure to arsenic in rats. *Food Chem Toxicol*, **44**: 1579‐ 1584.

Newsholme EA and Start C. 1981. **Regulation in Metabolism**. John Wiley and Sons. Chichester, UK.

Patra PH, Bandyopadhyay S, Kumar R, Datta BK, Maji C, Biswas S, et al. 2012. Quantitative imaging of arsenic and its species in goat following long term oral exposure. *Food Chem Toxicol*, **50:**1946–1950.

Prakash A, Khan S, Kumar D, Telang AG and Malik JK. 2014. Concurrent administration of curcumin mitigates arsenic – and chlorpyrifos – induced apoptosis in rat thymocytes. *Adv Anim Vet Sci*, **2**: 407 – 413.

Reitman S and Frankel S. 1957. A colorimetric method for the determination of serum glutamate–oxaloacetate and pyruvate transaminases. *Am J Clin Pathol*, **28**:56–63.

Saxena U, Witte LD and Goldberg IJ. 1989. Release of endothelial cell lipoprotein lipase by plasma lipoproteins and free fatty acids. *J Biol Chem*, **264**:4349–4355.

Sugihara N, Ohnishi M, Imamura M and Furuno K. 2001. Differences in antioxidative efficiency of catechins in various metal-induced lipid peroxidations in cultured hepatocytes. *J Health Sci*, **47:** 99-106.

US Environmental Protection Agency. 2001**. National Primary Drinking Water Regulations: Arsenic and Clarifications to Compliance and New Source Contaminants Monitoring**. Federal Register **40**:41–142.

World Health Organisation. 2011. **Guidelines for drinking–water quality**. Fourth edition. Edited by WHO. ISBN: 978 92 4 154815 1.

Zhang J, Pan X, Li N, Li X, Wang Y, Liu X, Yin X and Yu Z. 2014. Grape seed extract attenuates arsenic-induced nephrotoxicity in rats. *Exper Therap Med*, **7**: 260-266.

JJBS

Volume 9, Number 3, September .2016
ISSN 1995-6673
Pages 179 - 183

Jordan Journal of Biological Sciences

Effect of Prenatal Cigarette Smoke Exposure on Hematological Characteristics in Adult Rat Offspring

Jumah M. Shakhanbeh*

Department of Applied Biology, Faculty of Science, Tafila Technical University,Tafila, Jordan

Received: April 22, 2016 Revised: July 15, 2016 Accepted: July 29, 2016

Abstract

Maternal cigarette smoking during pregnancy is associated with many severe developmental abnormalities on the fetus. The present study investigates the hematological parameters of the blood of adult rat offspring exposed prenatally to Cigarette Smoke (CS). Pregnant albino rats were exposed to CS 3 times/day (each 15 minutes) or room air (controls) from gestational day 0 to parturition. Hematological parameters were measured for 3 month–old adult offspring. Prenatal CS exposure significantly increases total leukocyte counts, monocytes ($P < 0.05$), and lymphocytes ($P < 0.01$); and significantly decreases mean corpuscular volume of red blood cells ($P < 0.05$) in adult rat offspring as compared with controls. However, prenatal CS exposure had no significant effect on other hematological parameters, including red blood cells count, granulocytes, platelets, hemoglobin concentration, hematocrit, mean corpuscular hemoglobin, red cell distribution width, prothrombin time, mean platelet volume and platelet distribution width in adult rat offspring. These findings may indicate that cigarette smoking during pregnancy is associated with a significant increase in total leukocyte counts, lymphocytes and monocytes, and significant decreases mean corpuscular volume of red blood cells of adult rat offspring.

Keywords: Pregnant rats; prenatal cigarette smoke exposure; hematological parameters; adult rat offspring.

1. Introduction

Maternal cigarette smoking during pregnancy is a known risk factor for many severe pre- and postnatal developmental complications in the human fetus and infant. Prenatal CS exposure is the main risk factor in postnatal morbidity and mortality, intrauterine growth retardation, preterm delivery and sudden infant death syndrome (Andres and Day, 2000; Dempsey and Benowitz, 2001; Duncan et al., 2009). In addition, cigarette smoking during pregnancy can induce several prenatal complications, including spontaneous abortion, low birth weight, and increase in neonatal nucleated red blood cells (Blake et al., 2000; Voigt et al., 2006; Dollberg et al., 2000; Giovanni et al., 2013). CS is known to be a complex mixture of large number of highly toxic substances can lead to damage to neonates through active or passive smoking. Many of these metabolites, such as nicotine, cotinine, polycyclic aromatic hydrocarbons, metals and carbon monoxide can readily cross the placental barrier to the developing embryo and cause both prenatal and postnatal health consequences (Lambers and Clark 1996; Pichini et al., 2000). Some of these compounds were identified in the fetus blood and urine of newborns of smoking mothers (Pichini et al., 2000; Mamsen et al., 2010; Berlin et al., 2010; Giovanni et al., 2013), and in fetal blood of rodents (Carmines et al., 2003). These compounds can cause vasoconstriction to the uterine placental blood vessels (Donnenfeld et al., 1993; Albuquerque et al., 2004), resulting in serious reduction in oxygen supply and malnutrition to the developing fetus (Ganapathy et al., 1999). There also gestational exposure to CS stimulates the release of catecholamines from adrenal glands and sympathetic autonomic nerves which consequently limits the placental blood flow, causing intrauterine reduction in oxygen supply and under nutrition to the fetus (Ernest et al., 2001).

Several studies demonstrated that CS exposure or nicotine during gestational period is associated with significant permanent postnatal neurodevelopmental abnormalities in the activity of the central and peripheral nervous systems in adult offspring (Chen et al., 1998; Eugenin et al., 2008; Duncan et al., 2009; Bublitz and Stroud, 2012). This will result in severe deficits of many regulatory functions, including endocrinal (Fowler et al., 2009; Shields et al., 2009), cardiovascular (Blake et al., 2000; Xiao et al., 2008; Lawrence et al., 2008), respiratory (Gilliland et al., 2000; Singh et al., 2003; Pendleburg et al., 2008), reproductive (Jensen et al., 2005; Fowler et al., 2009; Mamsen et al., 2010),

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

autonomic neural control (Khan et al., 1994; Franco et al., 2000; Parslow et al., 2004; Schneider et al., 2008) and neurobehavioral outcomes (Pauly and Slotkin, 2008; Ernst et al., 2001). It is known that large majority of neurons in the cerebral cortex and other brain regions are generated during gestational period.

In addition, human studies have demonstrated significant immunomodulatory effects of maternal cigarette smoking during pregnancy on the immune system parameters by increasing in lymphocyte counts (Giovanni et al., 2013) and serum immunoglobulin levels (Cederqvist et al., 1984) in newborns of smoking mothers compared to those born to non-smoking mothers. In addition, other studies have described the immunosuppressive properties of maternal CS exposure by decreasing total leukocyte counts, lymphocytes and modulating the number and function of alveolar macrophages in neonates of smoking mothers (Mercelina-Roumans et al., 1996; Sopori and Kozak, 1998). Animal models have shown that gestational nicotine exposure down-modulates the immune function of adult offspring by suppressing lymphocyte responsiveness (Basta et al., 2000).

The aim of the present study is to investigate the effect of CS exposure during gestational period on hematological parameters of adult rat offspring.

2. Materials and Method

2.1. Animals

Two groups (each of 5 females) of young adult albino rats (10 weeks of age and weighing 150 - 160 g body weight) were used for mating. The first group was CS exposure, and the second group was controls. The animals were housed individually in plastic cages, and allowed to acclimatize for one week. The rats were provided with standard laboratory chow and water ad libitum except during CS exposure. Animal room was maintained at 20 – 25°C. Care and handling of the animals were performed in accordance with the international guidelines for the use of laboratory animals. During the mating period, the female rats were placed with fertile males overnight, and the next morning female rats were examined to have mated by the presence of sperms in the vaginal smears (considered gestational day 0). At the end of mating period, the males were removed, and the pregnant females were divided into two groups of 5 animals each: experimental CS exposure group and control group.

2.2. Cigarette Smoke Exposure

The pregnant females of CS exposure group were daily exposed to CS via whole-body inhalation (3 times/day, each 15 minutes) from gestational day 0 to parturition. CS was generated from the burning of filtered cigarettes using a smoking device (designed in Neurobiology laboratory, Mutah University) which pumps CS in the form of puffs (each 35 ml) at regular intervals (one puff/min, and puff duration of 2 seconds). There were no deaths or any other abnormal signs in the pregnant females associated with CS exposure. The control group was subjected to the same experimental conditions of the CS exposure group, but without exposure to CS. Food consumption and body weight gain of pregnant females of CS exposure group were similar to that of pregnant females of the control group. Newborn pups were kept with their mothers until weaning. After weaning, male and female offspring of CS exposure group and control group were separated and kept in the animal house. The hematological parameters of the blood were measured in 3 month–old adult offspring (150 – 170 g body weight) of both groups.

2.3. Hematological Investigation

Blood samples (5 ml) were collected by cardiac puncture under light ether anesthesia from adult rat offspring of CS exposure group (males = 15 and females = 11) and control group (males = 13 and females = 10). Immediately after blood collection, complete blood cell counts and hematological analysis were performed according to laboratory routine using automated hematology analyzer (Mythic) in Al-Karak hospital, Ministry of health. In addition, differential cell counts of leukocytes were performed manually.

2.4. Statistical Analysis

Statistical evaluations were performed by Student's t-test for independent samples. The level of significance of all tests was set at $P < 0.05$, and the results were expressed as means ± S.E..

3. Results

3.1. Cellular Components of the Blood of Adult Rat Offspring after Prenatal CS Exposure

The cellular components of the blood of adult rat offspring after prenatal CS exposure and controls are shown in Table 1. Prenatal CS exposure significantly increases the total leukocyte counts and monocytes ($P < 0.05$), and lymphocytes ($P < 0.01$) in male and female adult rat offspring as compared with controls. However, prenatal CS exposure had no significant effect ($P > 0.1$) on red blood cells count, granulocytes and platelets in male and female adult rat offspring as compared with controls.

3.2. Hematological Parameters of the Blood of Adult Rat Offspring after Prenatal CS Exposure

The hematological parameters of adult rat offspring after prenatal CS exposure and controls are shown in Table 2. Prenatal CS exposure significantly ($P < 0.05$) decreases the mean corpuscular volume of red blood cells in male and female adult rat offspring as compared with controls. However, prenatal CS exposure had no significant effect ($P > 0.1$) on other hematological factors including, hemoglobin, hematocrit, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, red cell distribution

width, prothrombin time, mean platelet volume and platelet distribution width in male and female adult rat offspring as compared with controls.

Table 1. Cellular components of the blood of adult rat offspring after prenatal CS exposure and controls.

Cellular component	Males		Females	
	CS exposure (n = 15)	Controls (n = 13)	CS exposure (n = 11)	Controls (n = 10)
RBCs ($X10^6$/ml)	8.31 ± 0.1	8.13 ± 0.11	7.71 ± 0.11	7.53 ± 0.33
Total Leukocytes ($X10^3$/ml)	9.92 ± 0.60*	6.81 ± 0.56	9.13 ± 0.64*	6.71 ± 0.60
Lymphocytes ($X10^3$/ml)	6.13 ± 0.43**	3.54 ± 0.40	4.78 ± 0.44**	3.60 ± 0.49
Monocytes ($X10^3$/ml)	1.44 ± 0.19*	0.97 ± 0.17	1.59 ± 0.33*	0.92 ± 0.13
Granulocytes ($X10^3$/ml)	2.34 ± 0.30	2.27 ± 0.21	1.32 ± 0.18	1.78 ± 0.27
Platelets ($X10^3$/ml)	611.50 ± 34.57	670.60 ± 24.12	657.18 ± 20.84	599.09 ± 15.14

Significantly different from controls (*P < 0.05, **P < 0.01).

Table 2. Hematological parameters of the blood of adult rat offspring after prenatal CS exposure and controls.

Hematological parameters	Males		Females	
	CS exposure (n = 15)	Controls (n = 13)	CS exposure (n = 11)	Controls (n = 10)
Hemoglobin (g/dl)	15.67 ± 0.25	15.50 ± 0.19	15.24 ± 0.26	14.77 ± 0.39
Hematocrit (%)	44.28 ± 0.43	44.54 ± 0.65	42.50 ± 0.56	42.65 ± 1.10
MCV (fL)	53.15 ± 0.52*	55.74 ± 1.15	54.37 ± 0.42*	56.81 ± 1.06
MCH (pg)	19.07 ± 0.43	19.1 ± 0.21	19.75 ± 0.23	19.68 ± 0.42
MCHC (g/dL)	34.80 ± 0.37	34.91 ± 0.64	35.64 ± 0.38	34.65 ± 0.38
RDW (%)	14.18 ± 0.27	15.16 ± 0.48	13.02 ± 0.53	13.6 ± 0.86
PT (%)	0.12 ± 0.01	0.16 ± 0.02	0.13 ± 0.01	0.13 ± 0.01
MPV (fL)	2.15 ± 0.13	2.59 ± 0.22	2.63 ± 0.34	2.98 ± 0.34
PDW (%)	15.75 ± 0.29	16.13 ± 0.30	16.48 ± 0.36	16.47 ± 0.30

Significantly different from controls (*P < 0.05). MCV: mean corpuscular volume, MCH: mean corpuscular hemoglobin, MCHC: mean corpuscular hemoglobin concentration, RDW: red cell distribution width, PT: prothrombin time, MPV: mean platelet volume, PDW: platelet distribution width.

4. Discussion

The present study investigates the hematological parameters of the blood of adult rat offspring exposed prenatally to maternal cigarette smoking. It reveals that prenatal CS exposure caused a significant increase in the total leukocyte counts, lymphocytes and monocytes in both male and female during late adult life. These findings are in agreement with a recent study which showed significant increase in lymphocyte counts in newborns of smoking mothers than those born of non-smoking mothers without significant change in the percentages of lymphocyte subpopulations (Giovanni et al., 2013). Therefore, it seems that maternal cigarette smoking during pregnancy has a long term stimulatory effect on the immune response in adult rat offspring. However, Schmid et al. (2007) showed that total leukocyte counts and lymphocytes were reduced in neonates of smoking mothers compared with those of non-smoking mothers. The decrease in total leukocyte counts might be due to the suppressive effect of nicotine on myeloid dendritic tissue of the bone marrow (Vassallo and Chen, 2004).

In addition, chronic fetal hypoxia and/or ischemia induced by carbon monoxide and nicotine of the CS can cause reduction of the uteroplacental blood flow during gestational period (Donnenfeld et al., 1993; Albuquerque et al, 2004) which may affect the development of hematopoietic tissues in bone marrow. This is supported by the findings of Dollbreg et al., 2000 who showed that infants born to women exposed to passive smoking during pregnancy have increased the number of circulating nucleated red blood cells. This is due to the increase in levels of erythropoietin hormone levels as Varvarigou et al. (1994) found in cord blood of infants born to smoking mothers. Since, it is known that this hormone has a stimulatory effect on bone marrow tissue after severe reduction in oxygen supply to body cells.

The present results show that prenatal CS exposure during pregnancy had no significant effect on red blood cell and platelet counts in male and female adult rat offspring. This is in agreement with the findings of Giovanni et al. (2013) who showed that red blood cell and platelet counts were similar in newborns of smoking mothers and non-smoking mothers. In addition, the present findings demonstrate that prenatal CS exposure caused a significant decrease in the mean corpuscular volume of red blood cells in male and female of adult rat offspring. However, other hematological factors, including, hemoglobin, hematocrit, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, red cell distribution width, prothrombin time, mean platelet volume and platelet distribution width, were not significantly affected after prenatal CS exposure of adult rat offspring..

5. Conclusion

The present study suggests that maternal cigarette smoking during pregnancy is associated with a significant increase in the total leukocyte counts, lymphocytes and monocytes, and significantly decreases the mean corpuscular volume of red blood cells of adult rat offspring. However, other hematological parameters were not significantly affected after prenatal CS exposure in adult rat offspring.

6. Acknowledgement

The present study was supported by a grant from Mutah University, Jordan.

References

Albuquerque CA, Smith KR, Johnson C, Chao R and Harding R. 2004. Influence of maternal tobacco smoking during pregnancy on uterine, umbilical and fetal cerebral artery blood flow. *Early Hum Dev*, **80**: 31-42.

Andres RL and Day MC. 2000. Perinatal complications associated with maternal tobacco use. *Semin Neonatal*, **5**: 231-241.

Basta P V, Basham K B, Ross W P, Brust M E and Navarro H A. 2000. Gestational nicotine exposure alone or in combination with ethanol down-modulates offspring immune function. *Int J Immunopharmacol*, **22**: 159-169.

Berlin I, Heilbronner C, Georgieu S, Meier C, Spreux-Varoquaux O. 2010. Newborns cord blood plasma cotinine concentrations are similar to that of their delivering smoker mothers. *Drug Alcohol Depend*, **107 (2**): 250-252.

Blake KV, Gurrin LC, Evans SF, Beilin LJ, Landau LI, Stanely FJ and Newnham JP. 2000. Maternal cigarette smoking during pregnancy, low birth weight and subsequent blood pressure in early childhood. *Early Hum Dev*, **57**: 137-147.

Bublitz MH, and Stroud LR. 2012. Maternal smoking during pregnancy and offspring brain structure and function: review and agenda for future research. *Nicotine Tob Res*, **14(4):** 388-397.

Carmines EL, Gaworski CL, Faqi AS and Rajendran N. 2003. *In utero* exposure to 1 R4F reference cigarette smoke: Evaluation of developmental toxicity. *Toxicol Sci*, **75**: 134-147.

Cederqvist LL, Eddey G, Abdel-Latif N and Litwin SD. 1984. The effect of smoking during pregnancy on cord blood and maternal serum immunoglobulin levels. *Am J Obstet Gynecol*, **148**: 1123-1126.

Chen WJ, Parnell SE and West JR. 1998. Neonatal alcohol and nicotine exposure limits brain growth and depletes cerebellar Purkinje cells. *Alcohol*, **15**: 33-41.

Dempsey DA and Benowitz NL. 2001. Risks and benefits of nicotine to aid smoking cessation in pregnancy. *Drug Safety*, **24:** 277-322.

Dollberg S, Fainaru O, Mimouni F, Shenhav M, Lessing J and Kupfermine M. 2000. Effect of passive smoking in pregnancy on neonatal nucleated red blood cells. *Pediatrics*, **106 (3)**: 34-39.

Donnenfeld A, Pulkkinen A, Palomaki GE, Knight GJ and Haddow JE. 1993. Simultaneous fetal and maternal cotinine levels in pregnant women smokers. *Amer J Obstet Gynecol*, **167**: 781-782.

Duncan JR, M, Myers M, Fifer WP, Yang M, Kinney HC, and Stark RI. 2009. Prenatal nicotine-exposure alters fetal autonomic activity and medullary neurotransmitter receptors: implications for sudden infant death syndrome. *J Appl Physiol*, **107(5)**: 1579-1590

Ernst M, Moolchan ET, Robinson ML. 2001. Behavioral and neural consequences of prenatal exposure to nicotine. *J Am Acad Child Adolesc Psychiatry*, **40**: 630–641.

Eugenin J, Otarola M, Bravo E, Coddou C, Cerpa V, Parada M, Llona E and Bernhardi R. 2008. Prenatal to early postnatal nicotine exposure impairs central chemoreception and modifies breathing pattern in mouse neonates: A probable link to sudden infant death syndrome. *J Neurosc*, **28(51)**: 13907-13917.

Fowler PA, Bhattacharya S, Gromoll J, Monteiro A and Oshaughnessy PJ. 2009. Maternal smoking and developmental changes in luteinizing hormone (LH) and the LH receptor in the fetal testis. *J Clin Endocrinol Metab*, **94**: 4688-4695.

Franco P, Chabanski S, Szliwowski H, Dramaix M and Kahn A. 2000. Influence of maternal smoking on autonomic nervous system in healthy infants. *Pediatr Res*, **47(2)**: 215-220.

Ganapathy V, Prasad PD, Ganapathy ME and Leibach FH. 1999. Drugs of abuse and placental transport. *Adv Drug Deliv Rev*, **38**: 99-110.

Gilliland FD, Berhane K, McConnell R, Gauderman WJ, Vora H, Rappaport EB, Avol E and Peters JM. 2000. Maternal smoking during pregnancy, environmental tobacco smoke exposure and childhood lung function. *Thorax*, **55**: 271-276.

Giovanni A, Gernot E, Andrea L, Christian S, Christian K, Peter H, Rudolf T, David B and Martina P. 2013. Maternal cigarette smoking and its effect on neonatal lymphocyte subpopulations and replication. *BMC Pediatr*, **13:** 57.

Jensen MS, Mabeck LM, Toft G, Thulstrup AM and Bonde JP. 2005. Lower sperm counts following prenatal tobacco exposure. *Hum Reprod*, **20**: 2559-2566.

Khan A, Groswasser J, Sottiaux M, Kelmanson J, Rebuffat E, Franco P, Dramaix M and Wayenberg JL. 1994. Prenatal exposure to cigarettes in infants with obstructive sleep apneas. *Pediatrics*, **93**: 778-783.

Lambers DS and Clark KE. 1996. The maternal and fetal physiologic effects of nicotine. *Semin Perinatol*, **20**: 115-126.

Lawrence J, Xiao D, Xue Q, Rejali M, Yang S and Zhang L. 2008. Prenatal nicotine exposure increases heart susceptibility to ischemia/perfusion injury in adult offspring. *J Pharmacol Exp Ther*, **324**: 331-341.

Mamsen LS, Lutterodt MC, Andersen EW, Skouby SO, Sorensen KP, Andersen CY and Byskov AG. 2010. Cigarette smoking during early pregnancy reduces the number of embryonic germ and somatic cells. *Hum Reprod*, **25(11)**: 2755-2761.

Mercelina-Roumans PE, Breukers R B, Ubachs J M and van Wersch J W. 1996. Hematological variables in cord blood of neonates of smoking and nonsmoking mothers. *J Clin Epidemiol*, **49**: 449-454.

Parslow PM, Cranage SM, Adamson TM, Harding R and Horne RS. 2004. Arousal and ventilator responses to hypoxia in sleeping infants: effects of maternal smoking. *Respir Physiol Neurobiol*, **140**: 77-87.

Pauly JR and Slotkin TA. 2008. Maternal tobacco smoking, nicotine replacement and neurobehavioral development. *Acta Pediatrica*, **97**: 1331-1337.

Pendlebury JD, Wilson RJ, Bano S, Lumb KJ, Schneider JM and Hasan SU. 2008. Respiratory control in neonatal rats exposed to prenatal cigarette smoke. *Am J Respir Crit Care Med*, **177**: 1255-1261.

Pichini S, Basagana XB, Pacifici R, Garcia O, Puig C, Vall O, Harris J, Zuccaro P, Segura J and Sunyer J. 2000. Cord serum cotinine as a biomarker of fetal exposure to

cigarette smoke at the end of pregnancy. *Environ Health Perspect*, **108**: 1079-1083.

Schmid JMP, Kuehni CE, Strippoli MP, Roiha HL, Pavlovic R, Latzin P, Gallati S, Kraemer R, Dahinden C and Frey U. 2007. Maternal tobacco smoking and decreased leukocytes, including dendritic cells, in neonates. *Pediatr Res*, **61**: 462-466.

Schneider J, Mitchell I, Singhal N, Kirk V and Hasan SU. 2008. Prenatal cigarette smoke exposure attenuates recovery from hypoxemic challenge in preterm infants. *Am J Respir Crit Care Med*, **178**: 520-526.

Shields B, Hill A, Bilous M, Knight B, Hattersley AT, Bilous RW and Vaidya B. 2009. Cigarette smoking during pregnancy is associated with alterations in maternal and fetal thyroid function. *J Clin Endocrinol Metab*, **94(2)**: 570-574.

Singh SP, Barrett EG, Karla B, Boroujerdi SR, Langley RL, Kurup V, Tesfaigzi Y and Sopori ML. 2003. Prenatal cigarette smoke decreases lung cAMP and increases airway hyperresponsivness. *Am J Respir Crit Care Med*, **168**: 342-347.

Sopori, ML and Kozak, W. 1998. Immunomodulatory effects of cigarette smoke. *J. Neuroimmunol*, **83**: 148-156.

Varvarigou A, Beratis NG, Makri M and Vagenakis AG. 1994. Increased levels and positive correlation between erythropoietin and hemoglobin concentrations in newborn children and mothers who are smokers. *J Pediatrics*, **124**: 480-482

Vassallo R and Chen L. 2004. Selective suppression of dendritic cell functions by cigarette smoke extract. *Chest*, **125**: 107S.

Voigt M, Hermanussen M, Witter-Backofen U, Fusch C and Hesse V. 2006. Sex-specific differences in birth weight due to maternal smoking during pregnancy. *Eu J Pediatrics*, **165**: 757-761.

Xiao D, Xu Z, Huang X, Longo LD, Yang S and Zhang L. 2008. Prenatal Gender-related nicotine exposure increases blood pressure response to angiotensin II in adult offspring. *Hypertension*, **51**: 1239-1247.

JJBS

Volume 9, Number 3, September .2016
ISSN 1995-6673
Pages 185 - 194

Jordan Journal of Biological Sciences

Genotypic Characteristics of Clinical and Non-Clinical Isolates of *Pseudomonas aeruginosa:* Distribution of Different antibiogram Profiles and Molecular Typing

Wedad M. Al-Haik[1], Anas A. Al-Mahbash[2], Abdullah Y. Al-Mahdi[3], May M.E. Mohamed[4], A.M. Al-Haddad[5]

[1]*Department of Biology, Faculty of Science, Hadhramout University, Al-Mukalla, Yemen.*

[2,3]*Biology Department, Faculty of Science, Sana'a University, Sana'a, Yemen.*

[4]*Department of Medical Microbiology and Immunology, Faculty of medicine, University of Alexandria, Alexandria, Egypt.*

[5]*Department of Medical Laboratories, College of Medicine and Health Sciences, Hadhramout University, Al-Mukalla, Yemen.*

Received: April 22, 2016 Revised: August 1, 2016 Accepted: August 8, 2016

Abstract

The present study aims to characterize *Pseudomonas aeruginosa* isolates in three hospitals in Hadhramout, Yemen. Two hundred samples were collected from patients, health workers staff hands and environmental samples and *P. aeruginosa* isolates were typed using antibiotyping and ERIC-PCR. The results showed that eleven yielded ERIC genetic patterns (PI-PXI) and size of amplified DNA bands size approximately ranged from 150-200 bp to 450-500 bp per sample, the highest rate of isolates from genetic patterns (ERIC-PCR) PI and PII was 23.5%. ERIC-PCR typing results showed horizontal transmission from patient to patient who had the same genetic patterns, isolates from patients linked to all isolates from environmental sources and staff hand samples which had the same genetic patterns, that indicated there was direct relationships between them. About 16 Antimicrobial Resistance Profile (ARP) including resistance ranged from 5 to 13 antimicrobial. Resistance pattern A1 (48.2%) isolates was the more frequent. Antibiogram typing showed a link between strains isolated from patients and environmental sources, but failed to show a direct relationships between patients and staff hands samples. ERIC-PCR typing was a more precise molecular technique than antibiogram methods and should be used for monitoring and determination of the sources of infection.

Keywords: Nosocomial Infection, *Pseudomonas aeruginosa*, Genetic Pattern, Antibiogram, ERIC-PCR.

1. Introduction

Nosocomial infections occur worldwide and affect both developed and resource-poor countries. Infections acquired in health care settings are among the major causes of death and increased morbidity among hospitalized patients (Tikhomirov, 1987). Pseudomonas aeruginosa is a ubiquitous bacterium and considered the fourth most commonly isolated nosocomial pathogen of all hospital-acquired infections (Todar, 2008).

It is widespread in natural environments and considered an opportunistic secondary pathogen for humans that is capable of causing a major nosocomial infections and broad spectrum of infections such as urinary tract, burn, respiratory tract, meningitis, chronic otitis media and otitis externa, pseudomonal endocarditis, septicemia, etc. (Singh et al., 2006; Yang et al., 2011). There is a high mortality rate associated with this pathogen, especially in immunocompromised patients, and excessive mortality and morbidity associated with inefficient empirical therapy, leading to complications during treatment (Estahbanati et al., 2002).

Typing of strains is essential for determining the epidemiology of nosocomial infections and aiding in the design of pathogen control methods (Pal et al., 2010). P. aeruginosa has been typed based on its phe¬notypic characteristics, such as serotyping, biotyping, bacteriophage typing, pyocin typing, and antimicrobial susceptibility testing (Pitt, 1988). Determination of antimicrobial profiles is another typing method used frequently as a supplemental epidemiological tool for strain differentiation of P. aeruginosa (Jamasbi and Proudfoot, 2008).

Due to the plasticity of the phenotypic characteristics, molecular techniques (which are inherently more stable than phenotypic characteristics) have gained popularity for strain differentiation and epidemiological studies of many organisms. Molecular techniques, such as Pulsed-Field Gel Electrophoresis (PFGE) and DNA fingerprinting, have been used for P. aerugi¬nosa typing in recent years (Hernández et al., 1997).

In Yemen, few studies on prevalence, biochemical identification and antibiotics sensitivity test for P. aeruginosa from clinical specimens were reported (El-Souny and Magaam, 2009; Yacin, 2009).

The present study was done the first time in Yemen to identify the bacteria to their molecular level and to detect the epidemiology and source of nosocomial infections by genotyping using molecular techniques and antibiogram.

Molecular typing of strains is essential to determine the epidemiology of nosocomial infections and aiding in the design and monitoring of pathogen control methods (Pal et al., 2010)..

2. Materials and Methods

The present study was done with cooperation betweenMicrobiologydivision–Biology Department- Faculty of Science- Sana'a University, Yemen and the Clinical Genomics Center- Faculty of Medicine, Alexandria University, Egypt.

The samples were collected and cultivated in Departments of Medical Microbiology in the National Center for Central Public Health Laboratories of Hadhramout, Yemen.

2.1. Study Samples

The samples were collected from patients, who stayed three days in hospitals before specimen's collection, staff hands and the environment samples that during the period from November 2013 to May 2014."

A total of 200 samples were collected were distributed as the following:

- 140 samples were taken from patients admitted to all hospitals, the source of the clinical isolates included wound and burn exudates, urine, sputum, blood, CSF, throat swab and ear secretions using sterile swabs. Blood samples were cultivated (2cc blood/ 10 ml brain heart infusion) then incubated at 37oC for 7–14 days. Subcultures were done every 48 hrs on blood agar plates (Mansour et al., 2013).
- 15 samples were collected from health workers staff hand swab at midday, by which time staff members had been in contact with patients for several hours.
- 45 environmental samples were collected from various sites of Intensive Care Units (ICU), Neonatal Intensive Care Unit (NICU), pediatric unit, endoscopy unit, Operation Theatres (OT) and delivery room, including suction apparatus tubing, respirators (artificial ventilation tubing), air condition outlet, endoscopes, antiseptic solutions and water tap. Surfaces were swabbed with sterile moisture cotton swab sticks and fluid samples were pipetted using sterile disposable plastic pipettes. All swab specimens were carried to microbiology laboratory by using cetrimide agar medium.

2.2. Isolation, Purification and Preservation of Pseudomonas aeruginosa

All specimens were cultured on the cetrimide agar which is considered the selective media to isolate P. aeruginosa strains and incubated at 37oC for 24 hrs (Fazeli et al., 2012). P. aeruginosa colonies were purified by streaking and sub-culturing on Nutrient agar plates until pure cultures were obtained, then transferred in glycerol and stored at -20oC (Guessas and Kihal, 2004).

2.3. Antibiogram

Antibiotic susceptibility tests were carried out by the Kirby- Bauer disk diffusion technique according to Clinical Laboratory Standard Institute guidelines (CLSI, 2014). The following antibiotics (OXOID and HIMEDIA) were used: Amikacin (AK 30 µg), Aztreonam (AT 30 µg), Ceftazidime (CAZ 30 µg), Gentamicin (GEN 10 µg), Imipenem (IPM 10 µg), Piperacillin (PI 100 µg), Piperacillin/ tazobactam (PIT 100/10 µg), Ciprofloxacin (CIP 5µg), Ceftriaxone (CRO 30 µg), Ampicillin (AMP 10 µg), Cefotaxime (CTX 30 µg), Tobramycin (TOB 10 µg), Chloramphenicol (C 30 µg) and Erythromycin (E 15 µg). The test medium was Mueller-Hinton agar was used for growing the lawn of culture of the strains (Rajput et al., 2012).

2.4. Molecular Technique

2.4.1. DNA Extraction

A single colony of each isolate was selected and subcultured aerobically on blood agar at 37 °C for 24 hrs. DNA extract was obtained by suspending a loopful of the bacteria in 50 µl of lysis solution in 0.5 ml Eppendorf tubes. An even distribution of the bacterial pellet and adequate turbidity was obtained by 5 minutes vortexing of the suspension.

Lysates were diluted 10 times by adding them to 450 µl of sterile distilled water; vortex was done for 2 minutes, and finally, centrifugation for 5 minutes at 5000 rpm. Five µl of the supernatant were used for PCR (Potrykus et al., 2014).

2.4.2. Polymerase Chain Reaction Technique

Typing by Enterobacterial Repetitive Intergenic Consensus PCR (ERIC-PCR) (PCR TECHNE Company) was carried out as follows. Bacterial DNA was amplified with each primer (Invitrogen; primer1 (Q4255F06), primer2 (Q4255FO7)): ERIC1R (5' to 3') (ATG TAA GCT CCT GGG GAT TCA C) and ERIC2 (5' to 3') (AAG TAA GTG ACT GGG GTG AGC G) using a standard reaction mixture (1.5 mM MgCl2, 2 mM primer, 2.5 U of AmpliTaq, and 100 ng of template DNA) [Invitrogen; PCR Super Mix (Cat. No. 10572-014)].

The reaction conditions were as follows: 1 cycle of initial denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 45 s, annealing at 58°C for 60 s, and DNA chain extension at 72°C for 45 s, and a final extension at 72°C for 10 min.

Amplicons were separated by electrophoresis on 1.4% agarose gels and stained with ethidium bromide (Biotium, 2007).

2.4.3. DNA Detection by Agarose Gel Electrophoresis

Amplicons and DNA molecular weight marker (Gene Ruler 100 bp) were separated by electrophoresis (HVD Vertriebs- GmbH) on 1.4 % agarose gels prepared in Tris-borate EDTA buffer and stained with 10µg/ml of ethidium bromide incorporated in the gel.

The gel was subjected to electrophoresis at 80 volts for 1 hr. The gel was observed over an ultraviolet transilluminator (SCIE-PLAS), and the presence or absence of bands in a gel lane was determined by visual inspection.

2.5. Statistical Analysis

Statistical analyses were performed using the statistical Package for Social Science (SPSS for windows, version 16) (SPSS Inc., Chicago, IL, U.S.A) were expressed by percentages and compared using the chi-squared test and ROC curve. The difference was regarded significant when ($P< 0.05$) and non significant when ($P>0.05$).

3. Results

3.1. Sample Type and Isolation Rate of P. aeruginosa

Our results showed that P. aeruginosa where found and isolated from the patients, health workers and environmental hospital samples and the total of the positive samples were 56 (28%) out of 200.

3.2. Distribution of P. aeruginosa Isolates

The number of positive isolated from patients was 42 (30%) and the highest isolation rate was (60%) from wounds and burns patients followed by sputum (26.7%), urine (22.2%), throat swabs(20%), ear secretions(18.8%), CSF(18.2%) and the lowest rate from blood(16.6%).

The isolation rate of P. aeruginosa from hospital environmental samples was (26.7%). The highest rate of isolated was from suction apparatus tubing 50% where is statically significant ($P< 0.05$), and 40% from endoscope, 25% from respirators, 25% from water tap, 12.5% from air condition outlet and 12.5% from antiseptic solutions. The positive culture of P. aeruginosa from staff hand samples were (13.3%) (Table1).

Table 1. Isolation rate of Pseudomonas aeruginosa from samples obtained from patients, hospital environment and staff hands

Source	No. of samples	No. of isolates	Percentage of positive culture(%)	Chi-square χ^2	*P*-value
Patient samples					
Wounds and burns	30	18	60	17.9	0.000
Sputum	30	8	26.7	0.03	0.86
Urine	27	6	22.2	0.52	0.47
Throat swabs	20	4	20	0.71	0.4
Ear secretions	16	3	18.8	0.74	0.39
CSF	11	2	18.2	0.56	0.46
Blood	6	1	16.6	0.39	0.53
Total	140	42	30	-	-
Environment samples					
Suction apparatus tubing	8	4	50	2.0	0.16
Endoscope	5	2	40	0.37	0.55
Respirators	8	2	25	0.04	0.85
Water tap	8	2	25	0.04	0.85
Air condition outlet	8	1	12.5	0.99	0.32
Antiseptic solutions	8	1	12.5	0.99	0.32
Total	45	12	26.7	-	-
Staff hand samples					
Total	15	2	13.3	1.73	0.19

Significant at p-value ≤ 0.05, No: Number

3.3. Risk Factors for Acquiring P. aeruginosa

The age of the whole sample of 140 patients ranged from months to > 60 years old, the rate of infection among males was (57.1%) whereas females (42.8%). The difference was not significant (P=0.91), and many of those patients were suffering from some chronic diseases (Table2). The risk factors for *P. aeruginosa* infection were in males (57.1%); the age group (> 40-60 years) (42.8%) and surgery disease (47.6%) and ICU (47.5%) (Figure 1).

Table 2. Age, Sex, and diseases risk factors for infection by *Pseudomonas aeruginosa*

Variable	n=140												*P*-value
Age [years]	Infected (n= 42) Sex						Non-infected (n=98) Sex						
	Male	%	Female	%	Total	%	Male	%	Female	%	Total	%	
0-1	2	4.7	-	-	2	4.7	7	7.1	5	5.1	12	12.2	
>1-20	5	11.9	3	7.1	8	19.0	12	12.2	10	10.2	22	22.4	0.51
>20-40	4	9.5	4	9.5	8	19.0	8	8.1	3	3.1	11	11.2	
>40-60	12	28.5	6	14.2	18	42.8	20	20.4	18	18.3	38	38.7	
> 60	1	2.3	5	11.9	6	14.2	8	8.1	7	7.1	15	15.3	
Total	24	57.1	18	42.8	42	-	55	56.1	43	43.9	98	-	0.91
Comorbid conditions													
Diabetes	7	16.6	6	14.2	13	30.9	11	11.2	8	8.2	19	19.4	0.82
Cancer	4	9.5	6	14.2	10	23.8	6	6.1	5	5.1	11	11.2	0.51
Hypertensi-on	8	19.0	5	11.9	13	30.9	10	10.2	9	9.2	19	19.4	0.62
Cardiac disease	5	11.9	4	9.5	9	21.4	6	6.1	3	3.1	9	9.2	0.63
Surgery	8	19.0	12	28.5	20	47.6	21	21.4	18	18.4	39	39.8	0.31

N: number of samples

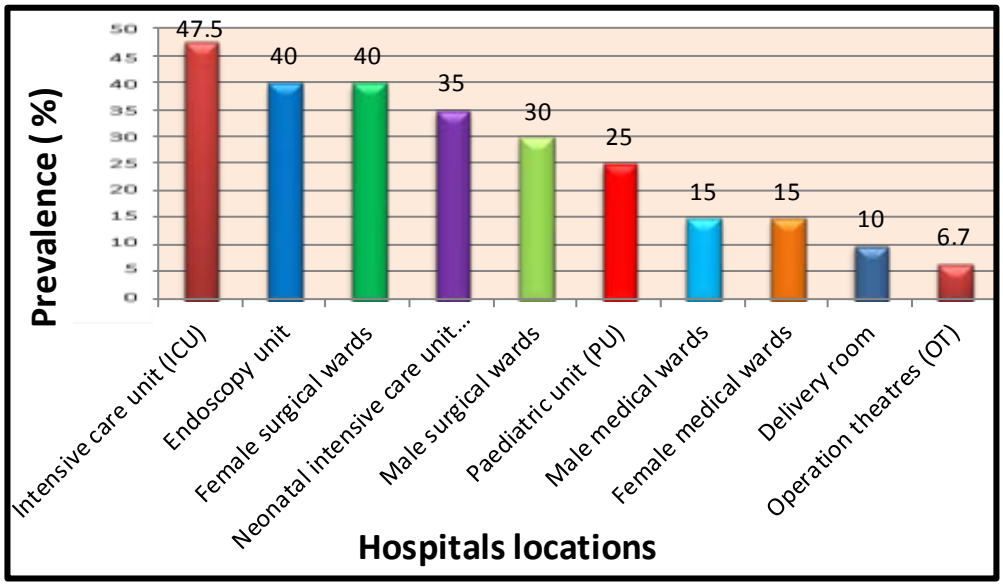


Figure 1. Prevalence of *Pseudomonas aeruginosa* in different locations of participating hospitals

3.4. Antibiotic Susceptibility

Among the 14 used antibiotics, the highest percentage of antibiotic resistance was to Ceftriaxone, Ampicillin, Cefotaxime and Chloramphenicol (100%) and low antibiotic resistance to Imipenem (5.4%) (Table 3).

Table 3. Antibiotic sensitivity tests *Pseudomonas aeruginosa* strains

Antibiotics	No. of esistant Strains	%	No. of ensitive Strains	%	No. of oderate Strains	%
Ceftriaxone	56	100	-	-	-	-
Ampicillin	56	100	-	-	-	-
Cefotaxime	56	100	-	-	-	-
Chloramphenicol	56	100	-	-	-	-
Erythromycin	55	98.2	-	-	1	1.8
Tobramycin	21	37.5	4	7.1	31	55.4
Ceftazidime	19	33.9	34	60.7	3	5.4
Amikacin	19	33.9	17	30.4	20	35.7
Piperacillin	15	26.8	31	55.4	10	17.9
Aztreonam	13	23.2	39	69.6	4	7.1
Ciprofloxacin	10	17.9	43	76.8	3	5.4
Gentamicin	10	17.9	35	60.7	11	19.6
Piperacillin/ tazobactam	6	10.7	45	80.4	5	8.9
Imipenem	3	5.4	53	94.6	-	-

3.5. Antibiogram

Table 4 shows the antibiogram of *P. aeruginosa* strains isolated from clinical, environment and staff hands samples, showing 16 ARP (antimicrobial resistance pattern) including resistance ranged from 5 to 13 antimicrobial. Resistance pattern A1 (48.2%) isolates was the more frequent.

3.6. Molecular Techniques

Out of 51 (91%) isolates *P. aeruginosa* were typeable by ERIC-PCR. The results showed that the size of amplified DNA bands ranged from (150-500) (base pair) with high genetic diversity and yielded 11 ERIC genetic patterns, with 1 to 3 bands (Figure 2).

The majority of strains were from pattern I and pattern II in rate of (24%) for each pattern whereas (18 %) from pattern III, (14%) from pattern IV, (3.9%) from pattern V, VI, VIII and IX and only one isolate (1.9%) from patterns VII, X and XI (Table 5).

Table 4. Antibiogram patterns of *Pseudomonas aeruginosa* strains isolated from Al-Mukalla Hospitals

ARP no.	ARP (Antibiogram)	No.of strains %	Samples
A1	CRO/CTX/C/E/AMP	27(48.2%)	Urine(3), wound and burn(7), Respirators(2), Throat swabs (4), sputum(4), staff hand, CSF, Endoscope, Suction apparatus tubing (2), Antiseptic solutions, Ear secretions
A2	CRO/CTX/C/E/AMP/CAZ	2 (3.6%)	wound and burn, staff hand
A3	CRO/CTX/C/E/AMP/TOB/AK	4 (7.1%)	CSF, Ear secretions, wound and burn, Air condition outlet
A4	CRO/CTX/C/E/AMP/CAZ/PI	2 (3.6%)	Suction apparatus tubing, Urine
A5	CRO/CTX/C/E/AMP/TOB/AK/CIP/AT/CAZ	2 (3.6%)	wound and burn, Water tap
A6	CRO/CTX/C/E/AMP/TOB/AK/CIP	2 (3.6%)	Water tap, wound and burn
A7	CRO/CTX/C/E/AMP/TOB/AK/CIP/PI	1 (1.8%)	Sputum
A8	CRO/CTX/C/E/AMP/TOB/AK/GEN/PI/CAZ/AT	4 (7.1%)	Suction apparatus tubing, wound and burn(2), Sputum
A9	CRO/CTX/C/E/AMP/TOB/AK/GEN/PI/CAZ/CIP/PIT/IPM	1 (1.8%)	Sputum
A10	CRO/CTX/C/E/AMP/PI/CIP/CAZ	2 (3.6%)	wound and burn, Blood
A11	CRO/CTX/C/E/AMP/TOB/CAZ/PI/GEN	1 (1.8%)	Sputum
A12	CRO/CTX/C/E/AMP/GEN/CAZ/AT	2 (3.6%)	wound and burn, Endoscope
A13	CRO/CTX/C/E/AMP/CIP/AK/TOB/GEN/PI/PIT	1 (1.8%)	wound and burn
A14	CRO/CTX/C/E/AMP/AK/TOB/GEN	1 (1.8%)	Urine
A15	CRO/CTX/C/E/AMP/CIP/AK/TOB/PIT/AT	1 (1.8%)	Ear secretions
A16	CRO/CTX/C/E/AMP/CAZ/PI/PIT/AT/TOB	3 (5.4%)	wound and burn(2), Urine

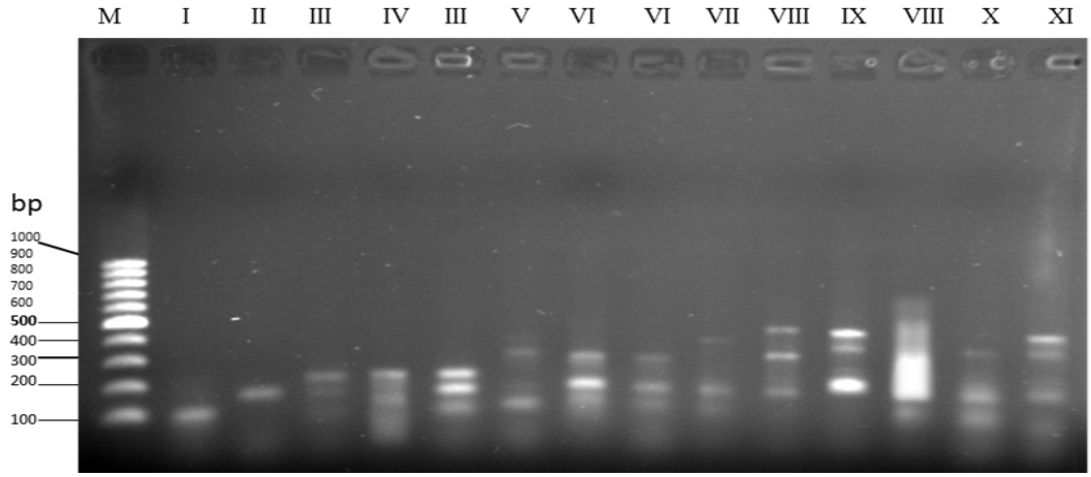


Figure 2. Different eleven patterns enterobacterial repetitive intergenic consensus (ERIC-PCR) of *Pseudomonas aeruginosa* strains

Table 5. (ERIC)-PCR patterns of *Pseudomonas aeruginosa* strains isolated from patients, hospital environment and staff hands

Genetic patterns No.	Bands No.	Size ofamplified DNA bands (bp)	Isolates No.	Isolates %
I	1	100- 150	12	23.5
II	1	150- 200	12	23.5
III	3	100-150, 200, 250-300	9	17.6
IV	2	100-150, 200-250	7	13.7
V	2	100-150, 300-350	2	3.9
VI	3	100-150, 200, 300	2	3.9
VII	2	150-200, 400	1	1.96
VIII	3	150-200, 300, 450-500	2	3.9
IX	3	150-200, 300-350, 400-450	2	3.9
X	2	100-150, 300	1	1.96
XI	3	100-150, 300, 400	1	1.96

3.7. Epidemiological Relationships of Genetic Patterns (ERIC PCR) of Pseudomonas aeruginosa Isolates

From ERIC-PCR typing methods P. aeruginosa strains (Table 6) and (Figure 3) showed horizontal transmission from patient to patient (ERIC-PI, PII, PIII, PIV, PVIII and PIX genetic patterns, respectively), probably from the hands of health care workers or environmental sources. Isolates from patients had ERIC-PI, PII, PIII, PIV and VI pattern linked to all isolates from environmental sources and staff hand samples that indicated that there were direct relationships between them.

ERIC-PCR typing gave epidemiological relationships among sites: suction apparatus tubing, respirators and staff hand samples that had the same patterns (ERIC-PII genetic patterns), also, it found links to suction apparatus tubing, endoscope, air condition outlet and antiseptic solutions (ERIC-PIII and PIV genetic patterns).

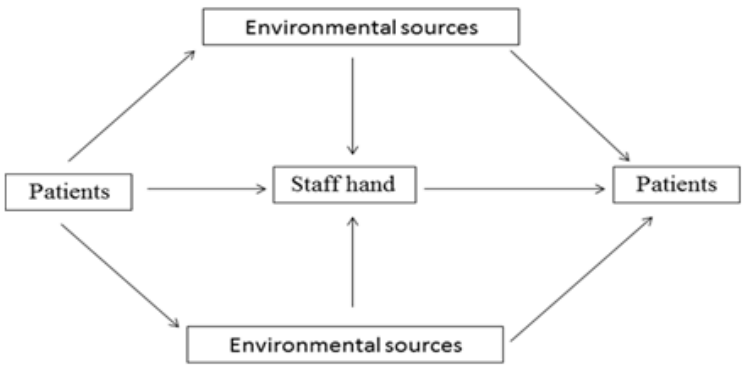


Figure 3. Diagram of epidemiological relationships between strains isolated from patients, environmental sources and staff hand using genotyping method

Table 6. Epidemiological relationships of genetic patterns (ERIC PCR) of *Pseudomonas aeruginosa* strains isolated from samples obtained from patients, the environment and staff hands

Clinical samples	Genetic patterns No.										
	PI	PII	PIII	P IV	P V	P VI	P VII	P VIII	P IX	P X	P XI
Wounds and burns	7	-	3	4	-	-	1	1	1	1	-
Sputum	2	-	2	-	2	-	-	-	-	-	-
Urine	-	4	-	1	-	1	-	-	-	-	-
Throat swabs	-	3	-	-	-	-	-	-	-	-	-
Ear secretions	1	-	-	-	-	-	-	-	-	-	1
CSF	-	-	-	-	-	-	-	1	1	-	-
Blood	1	-	-	-	-	-	-	-	-	-	-
Environment and staff hands samples											
Suction apparatus tubing	-	2	1	1	-	-	-	-	-	-	-
Endoscope	-	-	1	1	-	-	-	-	-	-	-
Respirators	-	1	-	-	-	1	-	-	-	-	-
Water tap	1	-	-	-	-	-	-	-	-	-	-
Air condition outlet	-	-	1	-	-	-	-	-	-	-	-
Antiseptic solutions	-	-	1	-	-	-	-	-	-	-	-
Staff hand samples	-	2	-	-	-	-	-	-	-	-	-
Total	12	12	9	7	2	2	1	2	2	1	1
%	23.5	23.5	17.6	13.7	3.9	3.9	1.9	3.9	3.9	1.9	1.9

P: Pattern

3.8. Distribution of Antibiogram According to the Chromosomal Patterns

The distribution of chromosomal patterns (ERIC-PCR) according to Antimicrobial Resistance Profile (ARP), 51 of P. *aeruginosa* isolated (Table 7) showed 16 ARP including resistance ranged from 5 to 13 antimicrobials.

The strains with ERIC-PCR pattern I 23.5% (the more frequent) were distributed in 10 different ARPs (A1, A3, A5, A6, A8, A9, A10, A11, A15 and A16), there was a statistically significant difference between antibiogram and genetic pattern (ERIC-PCR) in the rate of isolation of *P. aeruginosa* (*P*-value= 0.695) and (Chi-square χ^2 = 1.4).

Table 7. Distribution of chromosomal patterns (ERIC-PCR) in relation to antimicrobial resistance pattern (ARP) for 51 *Pseudomonas aeruginosa* strains isolated from clinical, environment and staff hands samples from Al-Mukalla Hospitals

ARP no.	ARP (Antibiotypes)	No. of strains (%)	Genotypes (DNA patterns)	Chi-square χ^2	*P*-value
A1	CRO/CTX/C/E/AMP	24(47.1%)	PI, PII(8), PIII(6),PVIII(2), PIV(4), P V (2),PVI		
A2	CRO/CTX/C/E/AMP/CAZ	2 (3.9%)	PII ,P IX		
A3	CRO/CTX/C/E/AMP/TOB/AK	4 (7.8%)	PI, PIII, PIX, P XI		
A4	CRO/CTX/C/E/AMP/CAZ/PI	2 (3.9%)	PII, P VI		
A5	CRO/CTX/C/E/AMP/TOB/AK/CIP/AT/CAZ	1(1.96%)	PI		
A6	CRO/CTX/C/E/AMP/TOB/AK/CIP	2 (3.9%)	PI (2)	1.40	0.695
A7	CRO/CTX/C/E/AMP/TOB/AK/CIP/PI	1(1.96%)	PIII		
A8	CRO/CTX/C/E/AMP/TOB/AK/GEN/PI/CAZ/AT	3 (5.9%)	PI, PIII, P IV		
A9	CRO/CTX/C/E/AMP/TOB/AK/GEN/PI/CAZ/CIP/PIT/IPM	1(1.96%)	PI		
A10	CRO/CTX/C/E/AMP/PI/CIP/CAZ	2 (3.9%)	PI (2)		
A11	CRO/CTX/C/E/AMP/TOB/CAZ/PI/GEN	1(1.96%)	PI		
A12	CRO/CTX/C/E/AMP/GEN/CAZ/AT	2 (3.9%)	PIV, P X		
A13	CRO/CTX/C/E/AMP/CIP/AK/TOB/GEN/PI/PIT	1(1.96%)	P IV		
A14	CRO/CTX/C/E/AMP/AK/TOB/GEN	1(1.96%)	PII		
A15	CRO/CTX/C/E/AMP/CIP/AK/TOB/PIT/AT	1(1.96%)	PI		
A16	CRO/CTX/C/E/AMP/CAZ/PI/PIT/AT/TOB	3 (5.9%)	PI, PII,P VII,		

4. Discussion

Analysis of the results indicated the occurrence of 28% *P. aeruginosa* infections in all hospitals, out of 30% strains were from clinical patient specimens. This is in agreement with rate reported in Egypt and Saudi Arabia by Mansour *et al.* (32.8%) and (30.0%), respectively, and in Saudi Arabia by Al Johani *et al.* (2010) (30.6%). The majority of samples (60%) were isolated from wounds and burns, similar results were obtained in Yemen (Yacin, 2009) and Saudi Arabia (Mansour *et al*., 2013). The high rate of *P. aeruginosa* from wounds and burn this may be due to the ability of *P. aeruginosa* to colonize in damaged skin of burned patients, where the first defense barrier against bacteria is lost (Marquart *et al*., 2005). Also, the high rate in this ward might be due to that *P. aeruginosa* has the ability to survive in different environments and it becomes an opportunistic pathogen for patients especially in the burn ward (Jamasbi and Proudfoot, 2008).

In the present study, the percentage of hospital environment samples were 26.7% strains and 13.3% (*P*-Value= 0.19) of strains belonged staff hand samples. Similar results were obtained in Egypt and Saudi Arabia (Mansour *et al*., 2013) that shown the rate of isolation from environment samples were 25.0% and 23.3%, respectively, and from staff hand samples were 10.0% and 6.7%, respectively. The isolation rate of *P. aeruginosa* from environmental samples was 26.7%, which is slightly higher than a previous study in Egypt (19.5%) (Gad *et al*., 2007). The higher rate of isolation from staff hands could be due to lack of compliance of health care workers to hand-washing practices.

The highest percentage of *P. aeruginosa* isolates was (50%) (*P*-Value= 0.16) from suction apparatus tubing, this may be explained by the failure of sterilization of suction apparatus tubing, this is similarly that found in Egypt and Saudi Arabia (Mansour *et al*., 2013) (57.1%) and (42.9%), respectively. However, these findings are different from those of a previous study in which the number of suction tubing infection was lower than other infection (Pal *et al*., 2010).

The analysis showed that the risk factors for *P. aeruginosa* infection were in male (57.1%) (*P*-Value = 0.91), and the age group of (> 40-60) years (42.8%) (*P*-value= 0.51), this is in agreement with rates in previous studies (Zavascki *et al*., 2005; Jamasbi and Proudfoot, 2008; Pal et al., 2010), and surgery disease (47.6%) (*P*-values= 0.31) (Zavascki *et al*., 2005), the rate of *P. aeruginosa* in surgery was higher than that reported from U.S.A (8%) during 1990-1996 (Qarah, 2008).

In the present work, the highest incidence of *P. aeruginosa* (47.5%) (*P*-value = 0.002) was found in ICU, which was followed by endoscopy unit and female surgical wards (40%), NICU (35%), male surgical wards (30%), PU (25%), male and female medical wards (15%), delivery room (10%) and OT (6.7 %); this is in agreement with Pall *et al.* (2010) and Bashir *et al*. (2011). The Gram-negative bacterium *P. aeruginosa* is frequently associated with hospital-acquired infections in ICUs (Driscoll *et al*., 2007). In this connection, intensive care patients are more prone to infection because of the debilitating effect of a prolonged hospitalization and instrumentation (Jarlier *et al*., 1996).

The most resistance antibiotics used were Ceftriaxone, Ampicillin, Cefotaxime and Chloramphenicol (100%), and the most effective antibiotic used was Imipenem (94.6%) (Fazeli *et al*., 2012; Sedighi *et al*., 2015). However, these findings are different from those of previous studies (Kamel *et al*., 2011; Adeyemi *et al*., 2015). The differences in antibiotic susceptibility in different regions could be attributed to the differences in patient population, the duration of hospitalization, cross-infection, and the dose and types of antibiotics (Rossello *et al*., 1992).

The 16 different ARPs observed in the strains isolated in the present study presented resistance to antimicrobials characterizing the analyzed strains as multi-drug resistant, similarly to Loureiro *et al.* (2002). Multiple factors may contribute to *P. aeruginosa* antibiotic resistance. The low outer membrane permeability and active efflux systems have been implicated in this process (Westbrock-Wadman *et al*., 1999). The mechanisms of acquisition of resistance include mutation, transmission of resistant plasmids, and production of inducible b-lactamase enzymes (Pagani *et al*., 2004).

In the present study from ERIC-PCR typing methods *P. aeruginosa* strains showed horizontal transmission from patient to patient (ERIC-PI, PII, PIII, PIV, PVIII and PIX genetic patterns, respectively), probably from the hands of health care workers or environmental sources (Mansour *et al*., 2013). Isolates from patients linked to all isolates from environmental sources and staff hand samples which had the same genetic patterns that indicated there was direct relationships between them. However, these findings indicate transmission of these stains by contamination during the use of medical instruments. A similar observation was described by Yorioka *et al.* (2010).

The strains with ERIC-PCR pattern I were distributed in 10 different ARPs (A1, A3, A5, A6, A8, A9, A10, A11, A15 and A16), the statistically significant was (*P*-value= 0.695) and (Chi-square χ^2 = 1.4).

Antibiogram typing methods explained the link between strains isolated from patients and environmental sources, but failed to show a direct relationship between patients and hands of health staff workers.

A significant decline in susceptibility of *P. aeruginosa* to β-lactams, aminoglycosides, and quinolones has been observed in many countries, including the United States (Livermore, 2002). Nosocomial outbreaks and the spread of MDR in different hospitals have also been documented (Bukholm *et al*., 2002). It appears that the frequency

and rate of resistance to individual antibiotics are different in different regions (Gales *et al*., 2001).

Our ERIC-PCR genotyping indicated the high diversity of *P. aeruginosa*, it was found that some isolates possessed a unique genotype and the most stains with similar genotypic characteristics (Jamasbi and Proudfoot, 2008).

The frequent phenotypic and genotypic changes could complicate the epidemiological studies of *P. aeruginosa* (Jamasbi and Proudfoot, 2008). Therefore, until a more precise molecular technique becomes available, each health care facility should establish a procedure for prompt identification, and perform phenotypic and genotypic analysis frequently, along with an effective infection-control measurement to prevent or reduce the spread of *P. aeruginosa* infections.

References

Adeyemi M F, Ako-Nai AK, Adejuyigbe E, Ebhodaghe IB, Osho OP, Oyeniyi TT and Kassim OO. 2015. Molecular characterization and antibiotic resistance profiles of bacterial isolates cultured from HIV seropositive patients. Iternational Medical Public Journals. *Arch Clin Microbiol*, **6**:2.

Al Johani SM, Akhter J, Balkhy H, El-Saed A, Younan M and Memish Z. 2010. Prevalence of antimicrobial resistance among gram-negative isolates in an adult intensive care unit at a tertiary care center in Saudi Arabia. *Ann Sau Med*, **30**: 364-369.

Bashir D, Thokar M A, Fomda B A, Bashir G, Zahoor D, Ahmad S. *et al*. 2011. Detection of metallo-beta-lactamase (MBL) producing *Pseudomonas aeruginosa* at a tertiary care hospital in Kashmir. *Afr J Microbiol Res*, **5**: 164-172.

Biotium, 2007. Product Information: GelRed™ Nucleic Acid Gel Stain, 10,000x in DMSO. *Biotium.com*. [cited 2007 March 31].

Bukholm G, Tannaes T, Kjelsberg AB. *et al*. 2002. An outbreak of multidrug resistant *Pseudomonas aeruginosa* associated with increased risk of patient death in an intensive care unit. *Infect Control Hosp Epidemiol*, **23**:441–446.

Clinical and Laboratory Standards Institute, 2014. Performance Standards for Antimicrobial Susceptibility Testing; Twenty-Fourth Informational Supplement (CLSI document M100-S24).

Driscoll JA, Brody SL and Kollef MH. 2007. The epidemiology, pathogenesis and treatment of *Pseudomonas aeruginosa* infections. *Drugs, Clin Inf Disease*, **67**:351-368.

El-Souny WA and Magaam S. 2009. Sensitivity of multi-drug resistant *Pseudomonas aeruginosa* isolated from surgical wounds infections to essential oils and plant extract. *World J Med Sci*, **4**:104-111.

Estahbanati H K, Kashani PP and Ghanaatpisheh F. 2002. Frequency of *Pseudomonas aeruginosa* serotypes in burn wound infections and their resistance to antibiotics. *Burns*, **28**:340-348.

Fazeli H, Akbari R, Moghim S, Narimani T, Arabestani M R and Ghoddousi A R. 2012. *Pseudomonas aeruginosa* infections in patients hospital means, and personnel's specimens. *J Res Med Sci*, **23**:332-336.

Gad GF, El-Domany R A, Zaki S and Ashour H M. 2007. Characterization of *Pseudomonas aeruginosa* isolated from clinical and environmental samples in Minia, Egypt: prevalence, antibiogram and resistance mechanisms. *J Antimicrob Chemother*, **60**:1010-1017.

Gales AC, Jones RN, and Turnidge J. 2001. Characterization of *Pseudomonas aeruginosa* isolates: Occurrence rates, antimicrobial susceptibility patterns, and molecular typing in the Global SENTRY Antimicrobial Surveillance Program, 1997–1999. *Clin Inf Disease*, **32**: S146-S155.

Guessas B and Kihal M. 2004. Characterization of lactic acid bacteria isolated from Algerian arid zone raw goats' milk. *Afr J Biotechnol*, **3**: 339-342.

Hernández J, Ferrus M A, Hernández M. *et al*. 1997. Arbitrary primed PCR fingerprinting and serotyping of clinical *Pseudomonas aeruginos*a strains. *FEMS Immunol Med Microbiol*, **17**: 37-47.

Jamasbi R J and Proudfoot E M. 2008. Phenotypic and Genotypic Characteristics of Clinical Isolates of *Pseudomonas aeruginosa:* Rate of Occurrence and Distribution of Different Serotypes, Antimicrobial Susceptibility Profiles, and Molecular Typing. *Sci Lab Med*, **39**: 155-161.

Jarlier V, Fosse T and Philipponm A. 1996. Antibiotic susceptibility in aerobic gram-negative bacilli isolated in intensive care units in 39 French teaching hospitals (ICU study). *Intensive Care Med*, **22**: 1057-65.

Kamel GM, Ezz eldeen NA, El-Mishad M Y and Ezzat RF. 2011. Susceptibility pattern of *Pseudomonas aeruginosa* against antimicrobial agents and some plant extracts with focus on its prevalence in different sources. *Global Vet*, **6**: 61-72.

Livermore DM. 2002. Multiple mechanisms of antimicrobial resistance in *Pseudomonas aeruginosa*: our worst nightmare. *Clin Inf Diseases*, **34**: 634-40.

Loureiro MM, de Moraes BA, Mendonça VL, Quadra MRR, Pinheiro GS and Asensi M D. 2002. *Pseudomonas aeruginosa*: Study of Antibiotic Resistance and Molecular Typing in Hospital Infection Cases in a Neonatal Intensive Care Unit from Rio de Janeiro City, Brazil. Mem Inst Oswaldo Cruz, Rio de Janeiro, 97(3): 387-394.

Mansour S, Eldaly O, Jiman-Fatani A, Mohamed M and Ibrahim E. 2013. Epidemiological characterization of *P. aeruginosa* isolates of intensive care units in Egypt and Saudi Arabia. *East Mediter Health J*, **19**: 71-80.

Marquart M, Caballero A and Chomnawang M. 2005. Identification of a novel secreted protease from *Pseudomonas aeruginosa* that cause corneal erosions. *Ophthalmol Vis Sci J*, **46**:10.

Pagani L, Mantengoli E, Migliavacca R *et al*. 2004. Multifocal detection of multidrug-resistant *Pseudomonas aeruginosa* producing the PER-1 extended-spectrum b-lactamase in northern Italy. *J Clin Microbiol*, **42**: 2523-2529.

Pal R, Rodrigues M and Datta S. 2010. Role of *Pseudomonas* in nosocomial infection and biological characterization of local strains. *J Biosci Tech*, **1**: 170-179.

Pitt TL. 1988. Epidemiological typing of *Pseudomonas aeruginosa*. *Eur J Clin Microbiol Inf Disease*,7: 238-247.

Potrykus M, Sledz W, Golanowska M, Slawiak M, Binek A, Motyka A, Zoledowska S, Czajkowski R and Lojkowska E. 2014. Simultaneous detection of major blackleg and soft rot bacterial pathogens in potato by multiplex polymerase chain reaction. *Ann App Biol*, **165**: 474-487.

Qarah S. 2008. *Pseudomonas aeruginosa* infections. Web Medicine clinical reference.

Rajput A, Prajapati B, Chauhan B, Shah A, Trivedi T and Kadam M. 2012. Prevalence of Metallo-betalactamases (MBL) producing *Pseudomonas aeruginosa* in a Tertiary care Hospital. *J Basic App Med Res*, **1**: 304-308.

Rossello J, Olona M, Campins M. *et al*. 1992. Investigation of an outbreak of nosocomial infection due to a multiple drug-resistant strain of *Pseudomonas aeruginosa*. *J Hos Inf*, **20**: 87-96.

Sedighi M, Safiri S, Pirouzi S, Jayasinghe H, Sepidarkish M and Fouladseresht H. 2015. Detection and determination of the antibiotic resistance patterns in *Pseudomonas aeruginosa* strains isolated from clinical specimens in Hospitals of Isfahan, Iran, 2012. *Scimetr*, **3**: e21133.

Singh A, Goering RV, Simjee S, Foley SL and Zervos MJ. 2006. Application of Molecular Techniques to the Study of Hospital Infection. *Clin Micribiol Rev*, **19**: 512-530.

Tikhomirov, E. (1987): WHO Programme for the Control of Hospital Infections. *Chemiotherapia*, **3**:148-151.

Todar E. 2008. Textbook of bacteriology.http://www.textbookofbacteriology.net.

Westbrock-Wadman S, Sherman DR, Hickey MJ. *et al*. 1999. Characterization of a *Pseudomonas aeruginosa* Efflux Pump contributing to aminoglycoside impermeability. *Antimicrob Agents Chemother*, **43**:2975-2983.

Yacin N. 2009.Studies on *Pseudomonas aeruginosa* Prevalent in the Main Hospitals in Sana'a City-Yemen. A thesis of M.Sc. Department of Biology, Faculty of Sciences, Sana'a University.

Yang L, Jelsbak L, Marvig RL, Damkiær S, Workman CT, Rau M.H. *et al*. 2011. Evolutionary dynamics of bacteria in a human host environment. *PNAS USA*, **108**:7481-7486.

Yorioka K, Oie S and Kamiya A. 2010. Microbial contamination of suction tubes attached to suction instruments and preventive methods. *Jap J Inf Diseases*, **63**:124-127.

Zavascki A P, Cruz RP and Goldani LZ. 2005. Risk factors for imipenem resistant *Pseudomonas aeruginosa*: a comparative analysis of two case-control studies in hospitalized patients. *J Hos Inf*, **59**: 96-101.

JJBS

Volume 9, Number 3,September .2016
ISSN 1995-6673
Pages 195 - 203

Jordan Journal of Biological Sciences

Vegetation Assessment of Okigwe Limestone Quarry Site at Okpilla in Etsako East Local Government Area, Edo State

Osazuwa Kingsley Omofomwan*, Bamidele, Joseph Femi and Oyanoghafo Osazee Osahon

University of Benin, Benin City, Nigeria

Received: June 3, 2016 Revised: August 8, 2016 Accepted: August 14, 2016

Abstract

The objective of the present study is to assess the impact of limestone quarrying on vegetation and document the flora associated with quarry. Three sites were adopted for this research work which were Site A (4 years quarry site), Site B (1 year and 6months quarry site) and Site C (Surrounding area with no quarry activities). Line transect method was employed and the plant species were identified and quantitative community characteristics were also assessed. The result obtained showed that at site A, B and C there were 49, 36 and 74 plants species, respectively, distributed into 18, 16 and 29 plant families, respectively. The most dominant species rich-families were Fabaceae, Poaceae, Asteraceae and Euphorbiaceae. Herbs were the most dominant plant habit and there were more native plant species in all the studied sites. As the dominance reduced and evenness increased the diversity increased. The Jaccard similarity index revealed that Sites A and B when compared had the highest value of 0.4167 indicating there were more similar in terms of plant species present. It is evident that quarrying activities in the area are detrimental to the vegetation. This finding would be useful while formulating the management plan for the area.

Keywords: Nigeria, limestone, vegetation, families, Jaccard similarity index.

1. Introduction

Limestones are sedimentary rocks primarily of calcium carbonate. Quarrying of different useful minerals, such as limestone, is an old technology that has been in existence since ancient past (Ekmekçi, 1993). The direct negative effects of mining activities can be an unsightly landscape, loss of cultivated land, loss of forest and pasture land, and the overall loss of production. The indirect effects can be multiple, such as soil erosion, air and water pollution, toxicity, geo-environmental disasters, loss of biodiversity, and ultimately loss of economic wealth (Xia and Cai, 2002). Suspended dust, generated from limestone quarries activities, blocks light in the atmosphere from reaching plants through air and also settles on plants and blocks sunlight by covering the stomata of plant leaves that needs to perform photosynthesis. Dust may have physical effects on plants such as blockage and damage to stomata, shading, abrasion of leaf surface or cuticle, and cumulative effects like drought stress on already stressed species (Banez et al., 2010). Limestone mining creates large disturbances that significantly impact soil, vegetation and fauna and result in habitat fragmentation and loss (Sort and Alcañiz 1996). Measures of vegetation structure provide information on habitat suitability, ecosystem productivity, and help predict successional pathways (Silver et al., 2004; Wang et al., 2004).

Information are lacking for the flora associated with limestone quarrying sites at Okigwe Village in Etsako East LGA. The outcome of the present study will therefore provide a source of data on the ecological impact of limestone quarrying in Okigwe village of Etsako East LGA. The data generated will be useful at the post mining phase, when the quarry site is abandoned or the deposit is exhausted. The research objective was to document the flora associated with limestone quarry sites in Edo State and to assess the impact of limestone quarrying on vegetation using phytosociological analysis.

2. Materials and Method

The present study area is bestowed with rich natural vegetation as well as large reserve of mineral resources. Okigwe Village in Okpella plays host to the Edo cement factory and many more solid mineral related factories. The present study area was divided into three sites which was designated as site A, B, and C. Site A represented excavated limestone quarry for a period of four years, Site B represented one and half years while Site C was the control

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

where limestone quarrying activities was absence. The use of line transect measuring 50m was employed which was placed 10 times each in the different sites studied. The species found in the line transect were identified with the aid of appropriate literatures, manual, checklist and botanical flora (Keay et al., 1964 ; Gill, 1992; Akobundu and Agyakwa, 1998; Etukudo, 2003 and Aigbokhan, 2014). Quantitative community characteristics, such as frequency, abundance and density, were determined. The data collected were subjected to the following ecological analysis: Importance Value Index (IVI), Importance and diversity indices were determined. Species diversity was computed using PAST Software to analyze different diversity indices. Similarity between communities based on species composition was determined by Jaccard similarity index.

The Shannon diversity index (H') was calculated using the following equation:

$$H' = \sum \left(\frac{ni}{n}\right) in \left(\frac{ni}{n}\right)$$

where, H = Shannon-Weaver index

ni = importance value index

N = total importance value of all species

The values of Shannon diversity index are usually found to fall between 1.5 and 3.5 and only rarely surpass 4.5 (Magurran, 1988).

Evenness (E') was calculated:

$$E' = \frac{H}{Hmax}$$

where Hmax is the maximum level of diversity possible within a given population, which equals ln(number of species). Magurran (1988) explained that E ' ranges normally between 0 and 1,

where 1 representing a situation in which all the species are equally abundant.

Simpson Dominance Index= (ni/N)^2

where, ni = importance value index

N = total importance value of all species

$$\text{Jaccard similarity index} = \frac{A}{\sum (A + B + C)}$$

where A = the common species between sites; B= the species of site 1 and C

= species of site 2.

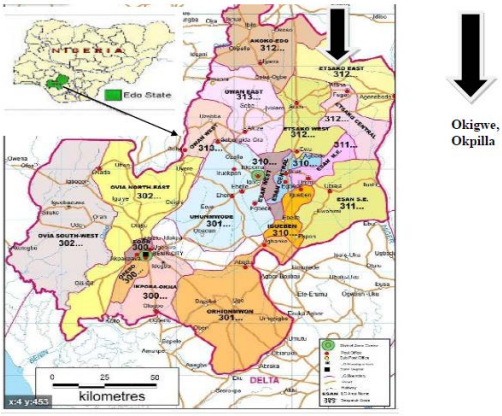


Plate 1: Map of the study area

3. Results

Forty-nine plants species were identified at site A in the studied area (Table 1), distributed in 18 plant families with Poaceae and Asteraceae having the most dominant with a value of 11 and 8 species represented, respectively (Figure 1). The data analyzed for species composition revealed that plants with importance value index (IVI) greater than 10 were Chromolaena odorata (L.) R. King &H. Robinson (13.293), Cyperus sp (32.779), Eurphorbia heterophylla L. (10.337), Sida acuta Burm. f. (56.991) and Syndrella nodiflora (L.) Gaert (11.826). Diplazium sammatii (Kuhn) C. Chr. was the only fern identified in this study. At site B, 36 plant species identified which were distributed into 16 plants families. Fabaceae and Poaceae were represented by 7 species each, followed by Asteraceae with 5 species (Figure 1). The data analyzed for species composition revealed that plants with importance value index (IVI) greater than 10 were Andropogon tectorum Schumach & Thonn (11.04), Aspilia africana (Pers.) C. D. Adams (11.04), Panicum maximum Jacq (14.57) and Spigelia anthelima L.(63.25). Seventy four (74) plants species were identified in site C, which were distributed in the 29 plant families with 1unknown family. The most dominant plant family in the site were Fabaceae (13 spp), Poaceae (11 spp), Asteraceae (7spp) and Euphorbiaceae (5 spp) (Figure 1). Plant with IVI greater than 10 were Manihot esculenta Crantz (10.04), Mimosa diplotricha C. Wright ex Sauvalle (13.25) and the unknown tree (24.85) with the other 70 plants in site C less than 10.

Table 1: Species composition of plants found in the 4 years old limestone quarry site A at Okigwe, Okpella. A=Abundance; D=Density; F= Frequency; RA=Relative abundance; RD= Relative density; RF=Relative frequency; IVI=Importance values index

S/No	Scientific Name	A	F	D	R A	RF	RD	IVI
1	*Acalypha fimbriata* L.	2.33	30	0.7	0.761	2.4	0.74	3.901
2	*Acroceras zizanoides* (Kunth) Dandy	3.33	30	1	1.088	2.4	1.05	4.538
3	*Ageratum conyzoides* L.	3	10	0.3	0.981	0.8	0.32	2.101
4	*Alchornea cordifolia (Schum. & Thonn.) Muell. Arg.*	8.67	30	2.6	2.834	2.4	2.74	7.974
5	*Andropogon tectorum* Schumach & Thonn.	5.33	30	1.6	1.742	2.4	1.68	5.822
6	*Aspilia africana* (Pers.) C. D. Adams	4	30	1.2	1.308	2.4	1.26	4.968
7	*Calopogonium mucunoides* Desv.	1.75	40	0.7	0.572	3.2	0.74	4.512
8	*Carica papaya* L.	1.5	20	0.3	0.49	1.6	0.32	2.41
9	*Centrosema pubscens* Benth	4.25	40	1.7	1.389	3.2	1.79	6.379
10	*Chromolaena odorata* (L.) R. King &H. Robinson)	13.5	40	5.4	4.413	3.2	5.68	13.293
11	*Cissus caesia* Afzel.	2	20	0.4	0.654	1.6	0.42	2.674
12	*Cleome viscosa* L.	3	20	0.6	0.981	1.6	0.63	3.211
13	*Conyza sumaturensis* (Retz.) E. Walker	8.5	20	1.7	2.779	1.6	1.79	6.169
14	*Crotalaria retusa* L.	3.67	40	2	1.199	3.2	2.11	6.509
15	*Cynodon dactylon* (L.) Pers.	1.75	40	0.7	0.572	3.2	0.74	4.512
16	*Cynodon nfuemlensis* Vanderyst	1.67	30	0.5	0.546	2.4	0.53	3.476
17	*Cyperus* sp.	74	10	7.4	24.189	0.8	7.79	32.779
18	*Daniellia oliveri* (Rolfe) Hutch & Dalziel	1	10	0.1	.327	0.8	0.11	1.237
19	*Desmodium triflorum* (L.) DC	4	10	0.4	1.308	0.8	0.42	2.528
20	*Diplazium sammatii* (Kuhn) C. Chr.	6	10	0.6	1.961	0.8	0.63	3.391
21	*Eleusine indica* (L.) Gaetn	5	30	1.5	1.634	2.4	1.58	5.614
22	*Emilia praetermissa* Milne-Redhead	4	10	0.4	1.308	0.8	0.42	2.528
23	*Euphorbia heterophylla* L.	17	20	3.4	5.557	1.6	3.58	10.737
24	*Euphorbia hirta* L.	4	10	0.4	1.308	0.8	0.42	2.528
25	*Euphorbia hyssopifolia* L.	1.5	20	0.3	0.49	1.6	0.32	2.41
26	*Gomphrena celosioides* Mart.	2	10	0.2	0.654	0.8	0.21	1.664
27	*Hibiscus suratensis* L.	2.5	40	1	0.817	3.2	1.05	5.067
28	*Hyptis suaveolens* (L.) Poit.	3.2	50	1.6	1.046	4	1.68	6.726
29	*Ipomoea triloba* L.	1	10	0.1	0.327	0.8	0.11	1.237
30	*Mariscus alternifolius* Vahl	1	10	0.1	0.327	0.8	0.11	1.237
31	*Mimosa pudica* Linn.	1	10	0.1	0.327	0.8	0.11	1.237
32	*Mitracarpus villosus* DC	4	10	0.4	1.308	0.8	0.42	2.528
33	*Nauclea latifolia* Sm.	1	10	0.5	0.327	0.8	0.53	1.657
34	*Oldenlandia corymbosa* L.	4.67	30	1.4	1.527	2.4	1.47	5.397
35	*Panicum maximum* Jacq	12.5	20	2.5	4.086	1.6	2.63	8.316
36	*Paspalum scrobiculatum* L.	4	40	1.6	1.308	3.2	1.68	6.188
37	*Phyllanthus amarus* Schum. & Thonn.	1	10	0.1	0.327	0.8	0.11	1.237
38	*Sacciolepis africana* C. E. Hubb. & Snowden	7.75	40	3.1	2.533	3.2	3.26	8.993
39	*Setaria barbata* (Lam.) Kunth	7	20	1.4	2.288	1.6	1.47	5.358
40	*Setaria pumila* (Poir.) Roem & Schult	7	10	0.7	2.288	0.8	0.74	3.828
41	*Sida acuta* Burm. f.	35.5	10	35.5	11.621	8	37.37	56.991
42	*Spigelia anthelmia* L.	1.75	40	0.7	0.572	3.2	0.74	4.512
43	*Sporobolus pyramidalis* P. Beauv	3.67	30	1.1	1.199	2.4	1.16	4.759
44	*Stachytarpheta jamaicensis* (Linn.) Vahl	2	10	0.2	0.654	0.8	0.21	1.664
45	*Synedrella nodiflora* (L.) Gaert	7.33	60	4.4	2.396	4.8	4.63	11.826
46	*Tridax procumbens* Linn.	2	10	0.2	0.654	0.8	0.21	1.664
47	*Typha domigensis* Pers.	2.8	50	1.4	0.915	4	1.47	6.385
48	*Urena lobata* L.	5	10	0.5	1.634	0.8	0.53	2.964
49	*Vernonia cinerea* (L.) Less	1.5	20	0.3	0.49	1.6	0.32	2.41

Table 2: Species composition of plants found in the 1 ½ yrs old limestone quarry site B at Okigwe, Okpella.
A=Abundance; D=Density; F= Frequency; RA=Relative abundance; RD= Relative density; RF=Relative frequency; IVI=Importance values index

S/N	Scientific Name	A	D	F	RA	RF	RD	IVI
1	*Acalypha fimbriata* L	1.75	0.7	40	2.17	3.57	2.19	7.93
2	*Alchornia cordifolia* (Schum & Thonn.) Muell. Arg.	1.33	0.4	30	1.65	2.68	1.25	5.58
3	*Andropogon tectorum* Schumach & Thonn.	3	1.2	40	3.72	3.57	3.75	11.04
4	*Aspilia Africana* (Pers.) C. D. Adams	3	1.2	40	3.72	3.57	3.75	11.04
5	*Calopogonium mucunoides* Desv	2.5	0.5	20	3.1	1.79	1.56	6.45
6	*Chromolaena odorata* (L.) R. King &H. Robinson)	2	0.8	40	2.48	3.57	2.5	8.55
7	*Cissus caesia* Afzel.	1.5	0.3	20	1.86	1.79	0.94	4.59
8	*Crotalaria retusa* L.	2.33	0.7	30	2.89	2.68	2.19	7.76
9	*Cynodon dactylon* (L.) Pers.	2	0.8	40	2.48	3.57	2.5	8.55
10	*Cyperus iria* L.	1.5	0.6	40	1.86	3.57	1.88	7.31
11	*Desmodium triflorum* (L.) DC	3	0.6	20	3.72	1.79	1.88	7.39
12	*Euphorbia hirta* L.	1.33	0.4	30	1.65	2.68	1.25	5.58
13	*Hyptis suaveolens* (L.) Poit.	1.67	0.5	30	2.07	2.68	1.56	6.31
14	*Ipomoea triloba* L.	2	0.6	30	2.48	2.68	1.88	7.04
15	*Laportea aestuans* (Linn.) Chew	1.5	0.6	40	1.86	3.57	1.88	7.31
16	*Ludwigia decurrens* Walter	2.67	0.8	30	3.31	2.68	2.5	8.49
17	*Mimosa pudica* Linn.	2.25	0.9	40	2.79	3.57	2.81	9.17
18	*Mitracarpus villosus* DC	1.5	0.3	20	1.86	1.79	0.94	4.59
19	*Mucuna pruriens* (L.) DC	1.33	0.4	30	1.65	2.68	1.25	5.58
20	*Panicum maximum* Jacq	2.43	1.7	70	3.01	6.25	5.31	14.57
21	*Paspalum scrobiculatum* L.	1.67	0.5	30	2.07	2.68	1.56	6.31
22	*Phyllanthus amarus* Schum. & Thonn.	1	0.2	20	1.24	1.79	0.625	3.655
23	*Sacciolepis africana* C. E. Hubb. & Snowden	2	0.2	10	2.48	0.89	0.625	3.995
24	*Scoparia dulcis* L.	2	0.2	10	2.48	0.89	0.625	3.995
25	*Securinega virosa* (Roxb ex Willd)Baill	1	0.1	10	1.24	0.89	0.625	2.755
26	*Senna siamea* (Lam) Irwin & Barbeby	2	0.4	20	2.48	1.79	1.25	5.52
27	*Setaria barbata* (Lam.) Kunth	1.67	0.5	30	2.07	2.68	1.56	6.31
28	*Solenostemon monostachyus* (P. Beau.) Brig.	2	0.8	40	2.48	3.57	2.5	8.55
29	*Spigelia anthelmia* L.	15	12	80	18.61	7.14	37.5	63.25
30	*Sporobolus pyramidalis* P. Beauv	2	0.6	30	2.48	2.68	1.88	7.04
31	*Synedrella nodiflora* (L.) Gaert	1.33	0.4	30	1.65	2.68	1.25	5.58
32	*Talinum triangulare* (Jacq.) Willd.	1	0.3	30	1.24	2.68	0.94	4.86
33	*Tephrosia bracteolata* Guill. & Perr.	2	0.2	10	2.48	0.89	0.625	3.995
34	*Tridax procumbens* Linn.	1	0.3	30	1.24	2.68	0.94	4.86
35	*Urena lobata* L.	1.67	0.5	30	2.07	2.68	1.56	6.31
36	*Vernonia cinerea* (L.) Less	2.67	0.8	30	3.31	2.68	2.5	8.49

Table 3: Species composition of plants found in the control site. A=Abundance; D=Density; F= Frequency; RA=Relative abundance; RD= Relative density; RF=Relative frequency; IVI=Importance values index

S/No	Scientific Name	A	D	F	RF	RA	RD	IVI
1	*Acalypha fimbriata* L.	3	0.6	30	1.96	1.39	1.36	4.71
2	*Acroceras zizanoides* (Kunth) Dandy.	2	0.2	10	0.65	0.93	0.45	2.03
3	*Ageratum conyzoides* L.	4	2.4	40	2.61	1.86	5.45	9.92
4	*Albizia adianthifolia* (Schumach.) W. Wight.	1	0.1	10	0.65	0.46	0.23	1.34
5	*Alchornea cordifolia* (Schum & Thonn.) Muell. Arg.	1.33	0.4	30	1.96	0.62	0.91	3.49
6	*Andropogon tectorum* Schumach & Thonn.	1	0.1	10	0.65	0.46	0.23	1.34
7	*Anthocleista djaloensis* A.Chev.	1	0.3	30	1.96	0.46	0.68	3.1
8	*Anthocleista vogelli* Planch.	2	0.2	10	0.65	0.93	0.45	2.03

9	*Aspilia africana* (Pers.) C. D. Adams	2	0.2	10	0.65	0.93	0.45	2.03
10	*Calopogonium mucunoides* Desv.	1	0.1	10	0.65	0.46	0.23	1.34
11	*Calotropis procera* (Aiton) R.Br.	2.33	0.7	30	1.96	1.08	1.59	4.63
12	*Carica papaya* L.	2	0.4	20	1.31	0.93	0.91	3.15
13	*Celosia argentea* L.	1.5	0.3	20	1.31	0.69	0.68	2.68
14	*Chromolaena odorata* (L.) R. King &H. Robinson)	4	1.2	30	1.96	1.86	2.73	6.55
15	*Citrus sinensis* (L.) Osbeck.	1	0.2	20	1.31	0.46	0.45	2.22
16	*Cleome viscosa* L.	1	0.1	10	0.65	0.46	0.23	1.34
17	*Cocos nucifera* L.	1	0.1	10	0.65	0.46	0.23	1.34
18	*Commelina erecta* L.	3	0.6	20	1.31	1.39	1.36	4.06
19	*Corchorus olitorius* L.	2	0.2	10	0.65	0.93	0.45	2.03
20	*Crotalaria retusa* L.	3	0.3	10	0.65	1.39	0.68	2.72
21	*Cynodon dactylon* (L.) Pers.	1	0.1	10	0.65	0.46	0.23	1.34
22	*Daniellia oliveri* (Rolfe) Hutch & Dalziel	1.25	0.5	40	2.61	0.58	1.14	4.33
23	*Desmodium scorpiurus* (Sw.)Desv.	3.33	1	30	1.96	1.54	2.27	5.77
24	*Desmodium triflorum* (L.) DC.	3	0.6	20	1.31	1.39	1.36	4.06
25	*Elaeis guineensis* Jacq.	2.5	0.5	20	1.31	1.16	2.27	4.74
26	*Eleusine indica* (L.) Gaetn	2.5	0.5	20	1.31	1.16	2.27	4.74
27	*Emilia praetermissa* Milne-Redhead	2.33	0.7	30	1.96	1.08	1.59	4.63
28	*Euphorbia hirta* L.	1	0.2	20	1.31	0.46	0.45	2.22
29	*Ficus sur* Forssk.	2.25	0.9	40	2.61	1.04	2.05	5.7
30	*Gomphrena celosioides* Mart.	2	0.4	20	1.31	0.93	0.91	3.15
31	*Havea brasiliensis* (A. Juss) Mull.Arg	12	1.2	10	0.65	5.57	2.73	8.95
32	*Hewittia sublobata* Linn.	2.5	0.5	20	1.31	1.16	2.27	4.74
33	*Hyptis suaveolens* (L.) Poit.	1.25	0.5	40	2.61	0.58	2.27	5.46
34	*Ludwigia decurrens* Walter	3	0.3	10	0.65	1.39	0.68	2.72
35	*Mangifera indica* L.	2	0.4	30	1.96	0.93	0.91	3.8
36	*Manihot esculenta* Crantz	9.5	1.9	20	1.31	4.41	4.32	10.04
37	*Mariscus alternifolius* Vahl	1	0.1	10	0.65	0.46	0.23	1.34
38	*Mimosa diplotricha* C. Wright ex Sauvalle	7.75	3.1	40	2.61	3.59	7.05	13.25
39	*Mimosa pigra* L.	2	0.4	30	1.96	0.93	0.91	3.8
40	*Mitracarpus villosus* DC	1	0.1	10	0.65	0.46	0.23	1.34
41	*Morinda lucida* Benth	1	0.1	10	0.65	0.46	0.23	1.34
42	*Mucuna pruriens* (L.) DC	3.75	1.5	40	2.61	1.19	3.41	7.21
43	*Musa paradisiaca* L.	2.5	1	40	2.61	1.16	2.27	6.04
44	*Musa sapientum* L.	1.67	0.5	30	1.96	0.77	0.91	3.64
45	*Mussaenda erythrophylla* Schum. & Thonn.	1.5	0.3	20	1.31	0.69	0.68	2.68
46	*Nauclea latifolia* Sm.	2.75	1.1	40	2.61	1.28	2.5	6.39
47	*Ocimum gratissimum* L	2	0.6	30	1.96	0.93	1.36	4.25
48	*Panicum maximum* Jacq	4	0.8	20	1.31	1.86	1.82	4.99
49	*Paspalum scrobiculatum* L.	2	0.2	10	0.65	0.93	0.45	2.03
50	*Psidium guajava* L.	2.25	0.9	40	2.61	1.04	2.05	5.7
51	*Sacciolepis africana* C. E. Hubb. & Snowden	2	0.2	10	0.65	0.93	0.45	2.03
52	*Scoparia dulcis* L.	1	0.1	10	0.65	0.46	0.23	1.34
53	*Securinega virosa* (Roxb ex Willd)Baill	1	0.1	10	0.65	0.46	0.23	1.34
54	*Senna siamea* (Lam) Irwin & Barbeby	2	0.2	10	0.65	0.93	0.45	2.03
55	*Setaria barbata* (Lam.) Kunth	2	0.6	30	1.96	0.93	1.36	4.25
56	*Setaria pumila* (Poir.) Roem & Schult	3	0.9	30	1.96	1.39	2.05	5.4
57	*Sida acuta* Burm. f.	2	0.2	10	0.65	0.93	0.45	2.03
58	*Solenostemon monostachyus* (P. Beau.) Brig.	5	2	40	2.61	2.32	4.55	9.48
59	*Spigelia anthelmia* L.	4	2	40	2.61	1.86	4.55	9.02
60	*Sporobolus pyramidalis* P. Beauv	2	0.2	10	0.65	0.93	0.45	2.03
61	*Sterculia tragacantha* Lindl.	1.5	0.6	40	2.61	0.69	1.36	4.66

62	*Synedrella nodiflora* (L.) Gaert	1	0.1	10	0.65	0.46	0.23	1.34
63	*Talinum triangulare* (Jacq.) Willd.	2	0.2	10	0.65	0.93	0.45	2.03
64	*Tephrosia bracteolata* Guill. & Perr.	2	0.1	10	0.65	0.93	0.23	1.81
65	*Tephrosia pedicelata* Baker	2	0.1	10	0.65	0.63	0.23	1.51
66	*Trema orientalis* (L.) Blume	2	0.4	20	1.31	0.93	0.91	3.15
67	*Tridax procumbens* Linn.	3.33	1	30	1.96	1.54	2.27	5.77
68	*Typha domigensis* Pers.	2	0.4	20	1.31	0.93	0.91	3.15
69	*UNKNOWN- TREE*	35	3.5	10	0.65	16.24	7.96	24.85
70	*Urena lobata* L.	1	0.1	10	0.65	0.46	0.23	1.34
71	*Vernonia cinerea* (L.) Less	1	0.1	10	0.65	0.46	0.23	1.34
72	*Vitex doniana* Sweet	1	0.1	10	0.65	0.46	0.23	1.34
73	*Zea mays* L.	11	1.1	10	0.65	5.1	2.5	8.25
74	*Zornia latifolia* Sm.	1	0.1	10	0.65	0.46	0.23	1.34

in all the studied sites, for liana it was only in site A and B while for fern it was only in site A. The phytogeographical status or profile shows that there were more native plant species than the cosmopolitan, exotic, pantropical and unknown species in all the studied sites (Figure 3). Pantropical species were not seen in site B.

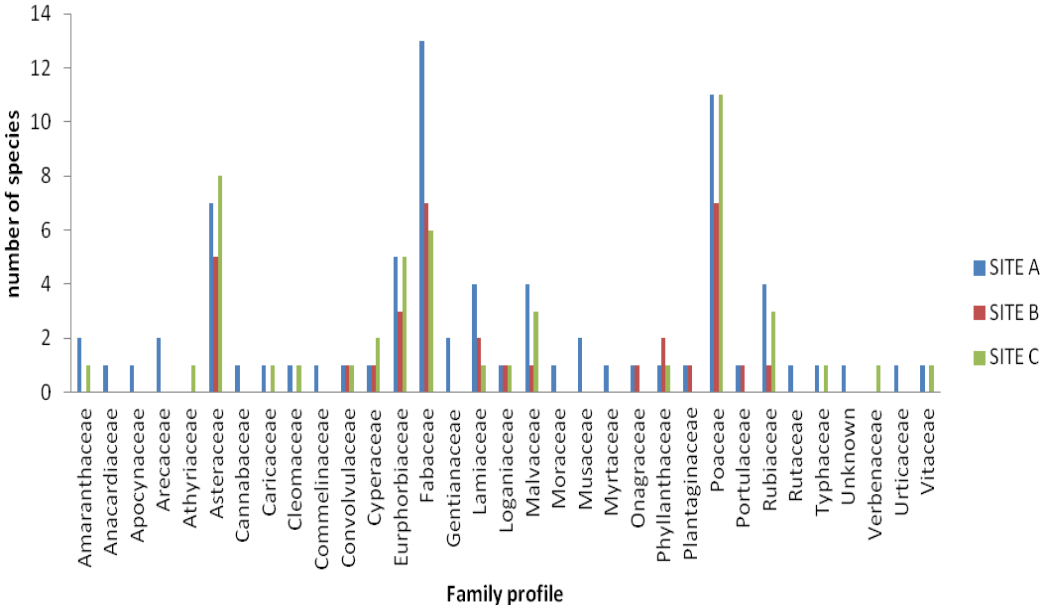


Figure 1: Family profile of plants at site A, B and C

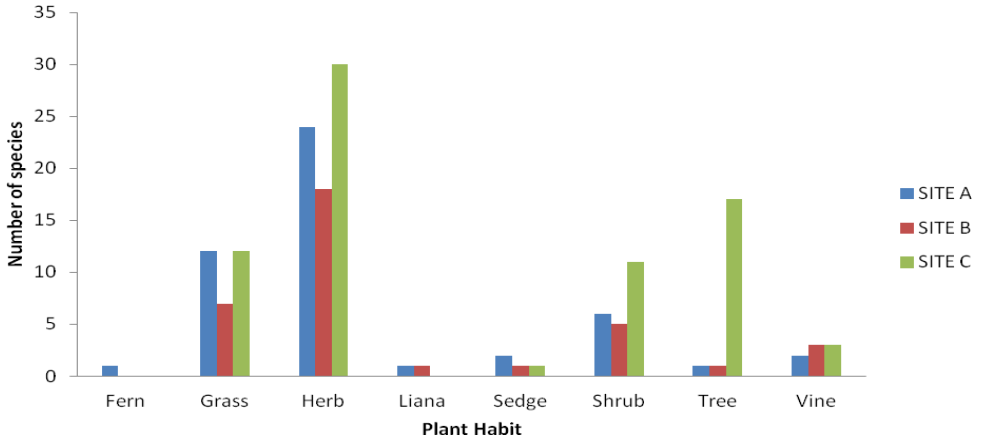


Figure 2: The abundance of plant types in limestone quarry sites at Okigwe

0

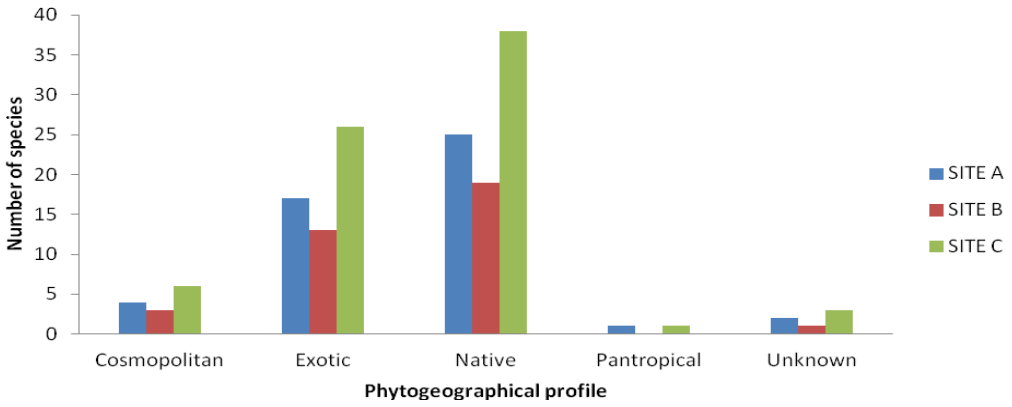


Figure 3: Phytogeographical profile of the different sites studied

The Simpson_1-D, Shannon-H, and Evenness were highest in site C with a value of 0.9703, 3.841, and 0.6291, respectively, Simpson_1-D, Shannon_H, Evenness, were lowest at Site A with values of 0.8413, 2.775, respectively. It was observed that as the dominance reduce and evenness increased the diversity increased as shown in Table 4 below. Presented below in Figure 4 is the Jaccard Similarity Index (JSI) for the different sites. It was observed that Site AB had more similar species than site AC and BC as evident in the high JSI value of 0.4167.

Table 4: Summary of some diversity indices

Diversity index	Site A	Site B	Site C
Taxa_S	49	36	74
Dominance_D	0.1587	0.1544	0.02974
Simpson_1-D	0.8413	0.8456	0.9703
Shannon_H	2.775	2.799	3.841
Evenness_e^H/S	0.3274	0.4562	0.6291

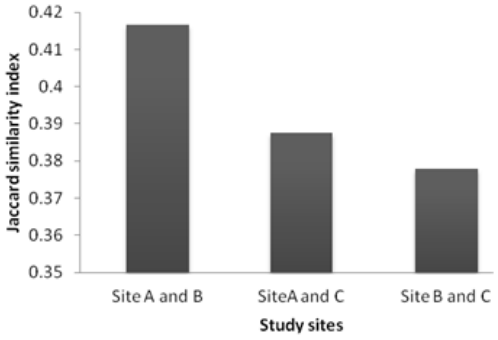


Figure 4: Jaccard similarity index for the three different sites

4. Discussion

Ecosystem disturbance are event or series of events that alters the relationship of organisms and their habitat in time and space. Many species of plants are disappearing at an unprecedented rate due to the direct or indirect effects of anthropogenic activities (Achard et al., 2002; Alford et al., 2007). Ecosystem disturbance by quarrying is an evitable fall out of industrialization and modern civilization which causes damage to the vegetation, hydrological relations and soil biological systems.

Result obtained from the present study sites indicates that a total of 90 plants species distributed into 33 plants families were identified. Xin-Sheng et al. (2012) reported a total vascular flora of 88 species belonging to 44 families and 76 genera was recorded from a limestone area in Cat Dua Island while Kumarasinghe et al. (2013) reported a total of 41 floral species in a commercial limestone quarrying site in Sri Lanka. In Site A, 49 plants species belonging to 44 genera and 18 families was identified. Site B had fewer species compared to that of sites A and C which had 36 plants species distributed into 36 genera and 16 families while site C had 74 species belonging to 29 families and 68 genera. The plant species composition in sites A and B were low when compared with site A. This is in line with the studies of Lyngdoh et al. (1992) and Sarma (2002)., while studying the impact of coal mining on the vegetation characteristics of the Nokrek Biosphere Reserve of Meghalaya outlined that the composition of vegetation reduces in the mined areas with that of the adjacent unmined areas. Lyngdoh et al. (1992) reported less number of species in the mine spoils of different ages to that the unmind sites. The differences in species composition could be attributed to the varying degree of quarrying activities. This is in agreement with the findings of Jha and Singh (1990), Das Gupta (1999), and Sarma (2002).

The most species rich families in the present study were Asteraceae, Eurphorbiaceae, Fabaceae and Poaceae but in contrast to these findings Xin-Sheng et al. (2012) reported that the dominant families of the flora were Euphorbiaceae, Papilionaceae, Moraceae, Rutaceae and Rubiaceae. Excavation of the ground for limestone minerals resulted in the reduction of tree richness in sites A and B. The most dominant plant types in the present study were the herbaceous species which was followed by grass in all the studied sites. This is in agreement with the findings of Sarma (2005) who reported that there was an increase in the number of herbaceous species and apparent decrease of tree species in the mined areas when compared with the

surrounding area. Other plant life form, identified in the present study, was a fern, climbers (liana, vine), sedges and shrubs.

It is suggested that the predominance of herbs and grasses compared to the trees and shrubs species in the quarry sites (A and B) imply that large disturbances contribute to habitat fragmentation and environmental degradation.

The study of the geographical distribution of plants is a very important aspect in vegetation science and based on this, the phytogeographical distribution of the plants in the present study was considered in the three sites studied, there were more native species, followed by exotic species. The native plant species contributed a high level of plant richness and diversity in the limestone quarrying sites. Also the high number of exotic species indicated their ability to survive in such a harsh environment.

The concept of 'Important Value Index (IVI)' has been developed for expressing the dominance and ecological success of any species, with a single value (Mishra, 1968). The IVI of plant species in site A as depicted in Table 1 shows that there were some plant with high values and some with very low IVI values. Some plants with high IVI in site A includes *Alchornia cordifolia* (7.974), *Chromolaena odorata* (13.293), *Cyperus* sp (32.779), *Euphorbia heterophylla* (10.737), *Panicum maximum* (8.316), *Sacciolepis africana* (8.993), *Sida acuta* (56.991) and *Synedrella nodiflora* (11.826). The data analyzed for species composition in Site B as depicted in Table 2 shows that the following plants *Andropogon tectorum Aspilia africana*, *Chromolaena odorata*, *Cynodon dactylon*, *Mimosa pudica*, *Panicum maximum* and *Spigelia anthelima* had high IVI values of 11.04, 11.04, 8.55, 8.55, 9.17, 14.57 and 63.25, respectively. In Site C, *Ageratum conyzoides* (9.92), *Solenostemon monostachyus* (9.48), *Spigelia anthelmia* (9.02) *Manihot esculenta* (10.04), *Mimosa diplotricha* (13.25) and the unknown tree (24.85) were the plants with high IVI values. When the IVI of all the plant species in the different sites was compared the *Spigella anthelmia* in Site B had the highest value of 63.25 followed by *Sida acuta* in Site A with a value of 56.991. The high importance value of *Spigella anthelmia* and *Sida acuta* in quarrying areas suggests their ability to grow and multiply in the disturbed environments and their dominance in the harsh conditions. Dominant species utilize their resources and have an extensive effect on the environmental conditions and when they are removed from the habitat, biotic and abiotic components of the habitat will change (Razavi et al., 2012).

Furthermore, in Site A plants species with low IVI values were *Daniellia oliveri* (1.237), *Ipomoea triloba* (1.237), *Mariscus alternifolius* (1.237), *Mimosa pudica* (1.237), *Tridax procumbens* (1.664). For Site B, they were *Scoparia dulcis* (3.995), *Securinega virosa* (3.995), *Senna siamea* (2.755) and *Tephrosia bracteolata* (3.995). While for Site C, they had relatively low IVI values (Table 3). The Simpson_1-D, Shannon-H, and Evenness were highest in site C with a value of 0.9703, 3.841 and 0.6291, respectively. As dominance_D reduced from Site A (0.1587) to Site B (0.1544) and then to Site C (0.9703), there was a gradual increase in the Simpson_1-D, Shannon_H, and Evenness_e^H/S indices. Shannon-Weaver index of diversity was much lower in the quarrying sites compared to control. This suggests dominance of one or two species in the quarrying sites. For example, in Site A and Site B *Sida acuta* (IVI: 56.991) and *Spigelia anthelmia* (IVI: 63.25) were the most dominant species, respectively. The Jacard similarity index obtained in this work revealed that Site A and B had the most similar plant species as seen by their high value of 0.4167. The Jacard similarity index for Site A and C was higher than that of Site B and C. It is suggested in the present study that the low similarity index of Site B and C is probably due to the fact that Site C was recently excavated, thus plants are trying to colonize the new harsh environment. Therefore, it indicates that as the excavated ground is left abandoned the plants growing in this area will gradual resemble that of the surrounding area. This was observed when Site A (4years) and C was compared for their similarity index.

5. Conclusion

It was found that the number of tree and shrub species decreased due to quarrying sites but the number of herbaceous species colonizing the quarrying areas increased. The present study led to the conclusions that phytosociological analysis can be used as important tools for predicting the impact of quarrying activities on vegetation. The information gathered on various aspects of vegetation and colonization of plants in quarrying areas would be helpful in revegetating the areas.

References

Achard F, Eva HD, Stibig H, Mayaux P, Gallego J, Ricahards T. and Malingreau J. 2002. Determination of deforestation rates of the world's humid tropical forests. *Science*, **297**: 999–1002.

Aigbokhan EI. 2014. Annotated checklist of vascular plants of southern Nigeria. A Quick Reference Guide to the Vascular Plants of Southern Nigeria: a systematic approach. University of Benin Printing Press. 345p.

Akobundu IO and Agyakwa CW 1998. A handbook of West African Weeds. International Institute of Tropical Agriculture. INTEC Printers, Ibadan. 564p.

Alford RA, Bradfield KS and Richards SJ 2007. Global warming and amphibian losses; The proximate cause of frog decline. *Nature*, **447**: 3-4.

Banez J, Ajaon SM, Bilolo JR and Dailyn JM 2010."Quarrying and Its Environmental Effects,"

Das Gupta S 1999. Studies on vegetal and microbiological processes in coal mining affected areas. Ph.D. Thesis. North_Eastern Hill University, Shillong. India.

Ekmekçi M 1993. Impact of quarries on karst groundwater systems, hydrogeological processes in Karst Terrenes, Proceedings of the Antalya Symposium and Field Seminar, October 1990, HS Publ. 3p.

Etukudo I 2003. Ethnobotany: Conventional and Traditional Uses of Plants. 1st Edn., The Verdict Press, Uyo, Nigeria. 24p.

Gill, LS 1992. Ethnomedical uses of plants in Nigeria. Uniben Press. 276p.

Jha AK and Singh JS 1990. Revegetation of mine spoils: Review and case study. *In*: Dhar, B. B. (ed.). Environmental Management of Mining Operations. Ashish Publishing House. New Delhi. pp. 300-326.

Keay RWJ, Onochie CFA and Stanfield DP 1964. Nigerian Trees. Nigerian National Press Ltd. 334p.

Kumarasinghe WAADG, Pradeep PIK, Peabotuwage RGATS, Wickramaarachchi S, Somarathne BNH, Perera UTI, Abeyawardane MR, Wijesinghe T and Karunarathna DMSS (2013). An attempt to reduce impacts of limestone quarries through biodiversity assessment and translocation: A case study at the Holcim Limestone Quarry Site in Puttalam, Sri Lanka. *Asian J Conserv Biol*, **2** (1): 3–20.

Lyngdoh T 1992. Community dynamics and edaphic changes in relation to coal mining in Jaintia Hills, Meghalaya. Ph.D. Thesis, North-Eastern Hill University, Shillong, India.

Magurran, AE. 1988. Ecological diversity and its measurement. Chapman and Hall, London.

Misra R 1968. Ecology WorkBook. Oxford and IBH Publishing Company, Calcutta.

Razavi SM, Mattaji A, Rahmani R and Naghavi F 2012. The Assessment of Plant Species Importance Value (SIV) in Beech (*Fagus orientalis*) Forests of Iran (A Case study: Nav District 2 of Asalem, Guilan Province). *Int Res J App Basic Sci*, **3**(2): 433-439.

Sarma K 2002. Coal mining and its impact on environment of Nokrek Biosphere Reserve, Meghalaya. Ph.D. Thesis. North-Eastern Hill University, Shillong. India.

Sarma K 2005 Impact of Coal Mining on Vegetation: A Case Study in Jaintia Hills District of Meghalaya, India. 85p.

Silver WL, Kueppers LM, Lugo AE, Ostertag R and Matzek V 2004. Carbon sequestration and plant community dynamics following reforestation of tropical pasture. *Ecol App* **14**:1115–1127.

Sort X and Alcañiz JM (1996). Contribution of sewage sludge to erosion control in the rehabilitation of limestone quarries. *Land Degrad Develop*, **7**: 69-76.

Wang J, Borsboom AC and Smith GC 2004. Flora diversity of farm forestry plantations in southeast Queensland. *Ecol Man Restor*, **5**, 43–51.

Xia HP and Cai XA 2002. Ecological restoration technologies for mined lands: a review. *China J App Ecol*, **13**(11):1471– 1477.

Xin-Sheng Q, Rong-Jing Z and Fu-Wu X 2012. A study on the flora and vegetation of Cat Dua Island, Northeastern Vietnam. *Pak J Bot*, **44**(4):1229-1232.

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. Ali Z. Elkarmi
The Hashemite University
P.O. Box 330127-Zarqa 13115 – Jordan
Telephone: 00 962 5 3903333 ext. 5157
Fax no. : 0096253903349
E. mail: jjbs@hu.edu.jo

المملكة الأردنية الهاشمية

المجلة الأردنية للعلوم الحياتية

مجلة علمية عالمية محكمة

تصدر بدعم من صندوق دعم البحث العلمي

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

ISSN 1995-6673