Jordan Journal of Biological Sciences (JJBS)

The **Jordan Journal of Biological Sciences (JJBS)** welcomes submissions of articles. The **JJBS** is to publish refereed, well-written original research articles, and studies that describe the latest research and developments in Biological Sciences. The JJBS is published quarterly and issued by Graduate Scientific Research Committee at the Ministry of Higher Education and Scientific Research and the Deanship of Scientific Research and Graduate studies at the Hashemite University, Zarqa, Jordan. The areas of interest include but are not limited to:

- Cell Biology
- Biochemistry
- Molecular Biology
- Genetics
- Immunology
- Plant Biology and Physiology
- Plant Taxonomy
- Animal Biology and Physiology
- Animal Diversity
- Mycology
- Bacteriology and Virology
- Ecology

Contributions in all areas at the interface of biology and other disciplines such as chemistry, physics, and mathematics are welcomed. Interested authors are encouraged to read "Instructions for Authors" while preparing their manuscripts. Send your contributions to:

Submission Address
Professor Naim S. Ismail
Deanship of Scientific Research and Graduate Studies
Hashemite University
P.O. Box 330127, Zarqa, 13115, JORDAN
Phone: +962-5-3903333 ext. 4147
E-Mail: jjbs@hu.edu.jo

Hashemite Kingdom of Jordan

Hashemite University

Jordan Journal of Biological Sciences

JJBS

An International Peer-Reviewed Scientific Journal

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

ISSN 1995-6673

INSTRUCTIONS FOR AUTHORS

All submitted manuscripts should contain original research not previously published and not under consideration for publication elsewhere. Papers may come from any country but must be written in English or Arabic with two abstracts, one in each language.

Research Paper: We encourage research paper of a length not exceeding 25 double-spaced pages. It should have a set of keywords (up to 6) and an abstract (under 250 words, unreferenced), followed by Introduction, Materials and Methods, Results, Discussion, Acknowledgments, and References.

Short Research Communication: It presents a concise study, or timely and novel research finding that might be less substantial than a research paper. The manuscript length is limited to 10 double-spaced pages (excluding references and abstract). It should have a set of keywords and an abstract (under 200 words, unreferenced), containing background of the work, the results and their implications. Results and Discussion Section should be combined followed by Conclusion. Materials and Methods will remain as a separate section. The number of references is limited to 60 and there should be no more than 4 figures and/or tables combined.

Reviews or mini-reviews should be authoritative and of broad interest. Authors wishing to contribute a manuscript to this category should contact the Editor-in-Chief. Reviews should describe current status of a specific research field with a balanced view. The length of the review manuscript should not exceed 50 double-spaced pages. Mini-reviews should not contain an exhaustive review of an area, but rather a focused, brief treatment of a contemporary development or issue in a single area. The length of the mini-review should not exceed 6 printed pages.

Reviewing the Manuscript:

A confirmation e-mail will be sent to the author upon receiving his manuscript. Please check your e-mail account frequently, because you will receive all important information about your manuscript through e-mail.

The accepted papers for publication shall be published according to the final date of acceptance. The editorial board reserves the right to reject a paper for publication without justification.

After the paper is approved for publication by the editorial board, the author does not have the right to translate, quote, cite, summarize or use the publication in other mass media unless a written consent is provided by the editor-in-chief as per JJBS policy.

Organization of Manuscript:

Manuscripts should be typewritten and double spaced throughout on one side of white typewriting paper with 2.5 cm margins on all sides. Abstract, tables, and figure legends should be on separate sheets. All manuscript sheets must be numbered successively. Authors should submit three copies of the paper and a floppy diskette 3.5 or a CD under (Winword IBM) or by e-mail.

Title page:

The title page of manuscript should contain title, author's names of authors and their affiliations, a short title, and the name and address of correspondence author including telephone number, fax number, and e-mail address, if available. Authors with different affiliations should be identified by the use of the same superscript on name and affiliation. In addition, a sub- field of submitted papers may be indicated on the top right corner of the title page.

Abstract:

The abstract should provide a clear and succinct statement of the findings and thrusts of the manuscript. The abstract should be intelligible in itself, written in complete sentences. Since JJBS is an interdisciplinary journal, it is important that the abstract be written in a manner which will make it intelligible to biologists in all fields. Authors should avoid non-standard abbreviations, unfamiliar terms and symbols. References cannot be cited in the Abstract.

Authors should submit with their paper two abstracts (English and Arabic), one in the language of the paper and it should be typed at the beginning of the paper before the introduction. As for the other abstract, it should be typed at the end of the paper on a separate sheet. Each abstract should not contain more than 250 words. The editorial board will provide a translation of abstract in Arabic language for non-Arabic speaking authors.

Introduction:

This section should describe the objectives of the study and provide sufficient background information to make it clear why the study was undertaken. Lengthy reviews of the past literature are discouraged.

Materials and Methods:

This section should provide the reader with sufficient information that will make it possible to repeat the work. For modification of published methodology, only the modification needs to be described with reference to the source of the method. Information regarding statistical analysis of the data should be included.

Results:

This section should provide hard core data obtained. Same data/information given in a Table must not be repeated in a Figure, or vice versa. It is not acceptable to repeat extensively the numbers from Tables in the text and give long explanations of the Tables and Figures. The results should be presented succinctly and completely.

Discussion:

The discussion should include a concise statement of the principal findings, discussion of the significance of the work, and appraisal of the findings in light of other published works dealing with the same or closely related object. Redundant descriptions of material in the Introduction and Results, and extensive discussion of literature are discouraged.

Acknowledgements:

If necessary, a brief Acknowledgements section may be included.

Citation:

Citation within text:

a. The reference is indicated in the text by the name of authors and year of publication between two brackets.
   Example: (Shao and Barker, 2007).

b. In the event that an author or reference is quoted or mentioned at the beginning of a paragraph or sentence or an author who has an innovative idea, the author's name is written followed by the year between two brackets.
   Example: Hulings (1986).

c. If the author's name is repeated more than once in the same volume and year, alphabets can be used. Example: (Khalifeh, 1994 a; Khalifeh, 1994 b).

d. If the number of authors exceeds two, the last name of the first author followed by et. al are written in the text. Full names are written in the references regardless of their number. Example (El-Betieha *et al*., 2008).

References list:

References are listed at the end of the paper in alphabetical order according to the author's last name.

a. Books:

Spence AP. 1990. **Basic Human Anatomy**. Tedwood City, CA, U.S.A.

b. Chapter in a book:

Blaxter M. 1976. Social class and health inequalities. In: Carter C and Peel J, editors. **Equalities and Inequalities in Health**. London: Academic Press, pp. 369-80.

c. Periodicals:

Shao R and Barker SC. 2007. Mitochondrial genomes of parasitic arthropods: implications for studies of population genetics and evolution. Parasit. **134**:153-167.

d. 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.

e. Theses and Dissertations:

El-Labadi SN. 2002. Intestinal digenetic trematodes of some marine fishes from the Gulf of Aqaba (MSc thesis). Zarqa (Jordan): Hashemite University.

f. In press articles:

Elkarmi AZ and Ismail NS. 2006. Population structure and shell morphometrics of the gastropod *Theodoxus macri* (Neritidae: Prosobranchia) from Azraq Oasis, Jordan. Pak. J. Biol. Sci. In press.

Authors bear total responsibility for the accuracy of references. Abbreviation of journal names should be given according to Chemical Abstracts or Biological Abstracts List of Sciences (BIOSIS).

Preparation of Tables:

Tables should be simple and intelligible without requiring references to the text. Each table should have a concise heading, should be typed on a separate sheet of paper, and must have an explanatory title. All tables should be referred to in the text, and their approximate position indicated on the margin of the manuscript. Ruling in tables, especially vertical or oblique line should be avoided.

Preparation of Illustrations:

Illustrations should be termed "Figures" (not "plates", even if they cover an entire page) and labeled with numbers. All figures should be referred to in the text and numbered consecutively in Arabic numerals (Fig. 1, Fig. 2, etc.). Scales in line drawings must be mounted parallel to either the top or side of the figures. In planning illustrations, authors should keep the size of the printed page in mind, making due allowance for the figure legend. The figures must be identified on the reverse side with the author's name, the figure number, and the orientation of the figure (top and bottom). The preferred location of the figures should be indicated on the margin of the manuscript. Illustrations in color may be published at the author's expense. The legends for several figures may be typed on the same page. Sufficient details should be given in the legend to make it intelligible without reference to the text.

JJBS
Jordan Journal of Biological Sciences

JJBS

Jordan Journal of Biological Sciences

Volume 2, Number 2, June 2009
ISSN 1995-6673
Pages 55 - 62

Evaluation of Hexaploid Wheat Varieties for Making Bread by High Molecular Weight (HMW) and Low Molecular Weight (LMW) Analysis

Nawroz Abdul-razzak Tahir*

University of Sulaimanyah, College of Agriculture, Bakrajo, Sulaimanyah, Iraq

Abstract

Knowledge of composition of high molecular weight glutenin subunits (HMW-GS) and low molecular weight glutenin subunits (LMW-GS) and their associations with bread making and quality will contribute to genetically improving processing quality of bread wheats in Iraq. Six bread wheat varieties (Tammuz, Aras, Rabia, Cham 4, Cham 6 and Costantino) were conducted to detect the allelic variation at Glu-1 and Glu-3 loci by SDS-PAGE electrophoresis and to understand their effects on dough properties. Important methods applied for the breeding of bread-quality wheat (*Triticum aestivum* L.) consist of small-scale bread-quality tests for the determination of the grain protein content, SDS-sedimentation volume, thousand kernel weight and kernel diameter. The thousand kernel weight and SDS-sedimentation volume showed relatively higher significant among the varieties, whereas the flour yield showed no significance difference. The results of SDS-PAGE indicate that subunits/alleles 1 and null at Glu-A1, 7, 20 and 7+9 at Glu-B1, 2+12 and 5+10 at Glu-D1, allele a at Glu-A3, alleles d and m at Glu-B3 and alleles j and k at Glu-D3 in bread wheat varieties. The lowest frequency of subunit 7+9 was found in variety Tammuz. On the other hand, the variety Tammuz showed the highest value of score quality and the varieties Cham 4 and Cham 6 had the lowest value of score quality. Genetic diversity of wheat was evaluated by constructing the dendrogram for high molecular weight (HMW) and low molecular weight (LMW) gluten subunit bands.

الملخص

ان معرفة الغلوتينين ذات الاوزان الجزيئة الصغيرة والاوزان الجزيئة الكبيرة وارتباطاتها مع صناعة الرغيف ونوعيتة ستسهم في المعالجة وراثيا لتحسين نوعية الخبز والحنطة في العراق. و قد استخدمت طريقة الفصل الكهربائي لهلام كبريتات دوديكل الصوديوم متعدد الأكريلامايد لفصل جزيئات الغلوتينين ذات الوزن الجزيئي الصغير والوزن الجزيئي الكبير وللكشف عن تباين الأليلات فى المواقع Glu-1 و Glu-3 وفهم تأثيرها على صفات العجين في مختلف اصناف الحنطة الناعمة: تموز، آراس ، ربيعة، شام 4, شام 6 و كوستانتينو. ان أهم الأساليب المتبعة لدراسة نوعية الخبز والحنطة هي اختبارات الجودة لتحديد محتوى البروتين، حجم الترسبات للبروتين ، وزن الألف حبة, الطحين الناتج, وقطر الحبة. ان النتائج برزت فروق معنوية بين الاصناف من الحنطة بالنسبة لوزن الألف حبة وحجم الترسبات للبروتين، في حين لم تظهر اي الفروقات معنوية بين الاصناف بالنسبة للطحين الناتج. وتشير النتائج إلى وجود الأليلان 1 ولاغ (Null) في موقع Glu-A1 و الأليلات 7, 20 ,7+9 في موقع Glu-B1 و ألأليلات 2+12 و 5+10 في موقع Glu-D1 والأليل a في موقع Glu-A3 والأليلان d و m في موقع Glu-B3 والأليلان j و k في موقع Glu-D3. وان الغلوتينين ذو الأليل 7+9 عثر عليها فقط في الصنف تموز. و في المقابل، اظهر الصنف تموز أعلى قيمة للجودة في تصنيع الخبز في حين أعطت الاصناف شام 4 وشام 6 أدنى قيمة للجودة فى تصنيع الخبز. كما ان الاختلاف الوراثي للحنطة من حيث الغلوتينين درس عن طريق تكوين الرسم الشجري.

Keywords: Bread Wheat; Wheat Quality; HMW and LMW Of Glutenin; SDS-PAGE (Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis).

1. Introduction

Wheat grain storage proteins are composed of 2 major fractions, gliadin and glutenin. Glutenin consists of both high-molecular-weight (HMW) and low-molecular weight (LMW) subunits. The HMW-glutenin subunits (HMW-GS) are encoded by Glu-A1, Glu-B1, and Glu- D1 on the long arm of chromosomes 1A, 1B and 1D, respectively (Payne et al., 1980). The LMW-glutenin subunits (LMW-GS) are encoded by Glu-A3, Glu-B3, and Glu-D3 on the short arm of these chromosomes (Gupta et al., 1990). Glutenin subunits were also classified into A (HMW-GS), B and C (LMW-GSs) subunits based on their mobility in SDS-PAGE analysis (Gupta et al., 1990). These glutenin subunits are polymerized by intermolecular disulfide bonds, which play a major role in the rheological properties of wheat flour doughs. It has been shown that allelic variations of HMW-GSs and LMW-GSs affect dough properties in various wheat cultivars (Gupta et al.,

* Corresponding author. nawrozbiology@gmail.com.

1991; Gupta et al., 1994; Khelifi et al., 1992; Nagamine et al., 2000; Payne et al., 1987a; Payne et al., 1987b). The HMW glutenins represents 5-10% of the total grain protein. The HMW glutenins are further subdivided into allelic pairs on 1B and 1D and a single subunit on 1A and each of these subunits influences wheat flour and dough quality. The role of individual LMW-GSs is, however, much less well characterized than that of HMW-GSs, because large numbers of the subunits display similar mobilities in SDS-PAGE analysis.

While the HMW glutenins are the major determinants of bread quality, LMW glutenins and gliadins are also important. Genes encoding the LMW glutenins are present on the short arm of chromosome 1A, 1B, and 1D.The LMW glutenins are one-third of the total seed protein and 60% of the total glutenins. The HMW and the LMW glutenins form extensive disulphide linked polymers that influence the dough quality. The LMW glutenins form aggregates may be important for dough strength. The cysteine residues in the LMW structure helps to separate two different HMW polymer-building subunits. The chain extenders (having two or more cysteine residue) allow the formation of stronger dough's, while chain terminators have the opposite effect (Greenfield et al*.,* 1998; Masci et al., 1998). The chain extender proteins have increased strength and stability due to the longer repetitive domains. The polypeptides with single cysteine residue have decreased dough strength and stability as they act as chain terminators in the glutenin polymers. The reduction in proportion of LMW glutenins, results in dough properties shifting towards greater strength due to an increase in the HMW/LMW glutenin ratio (MacRitchie and Lafiandra 2001; Lawerence et al., 1998). The increase in the polymeric proteins results in a stronger dough strength that is good for bread quality. In contrast the dough mixing strength is reduced in deletion lines missing the HMW glutenins. An increase in the amount of polymeric protein and better flour performance has also been demonstrated (Ciaffi et al., 1995; Rogers et al., 1997; Lafiandra et al., 1998). The use of registered crop varieties makes their expeditious identification important; its significance is increased by the diversity of varieties in many important traits. Each variety is characterized by a specific set of traits that determine its use.

Gliadins and glutens are genetic markers allowing the expeditious and objective identification of a variety, determination of its genetic constitution, and determination of some important characteristics and traits. Genetic diversity is the basis for successful crop improvement and can be estimated by different methods such as morphological traits, end-use quality traits, and molecular markers (Fufa et al., 2005). The present study was undertaken to evaluate the quality and genetic diversity in gluten-subunits in six wheat varieties using SDS-PAGE.

2. Materials and Methods

2.1. Plant Sample

Grains of wheat varieties were collected from the department of Agriculture of Sulaimanyah. The bread wheat varieties (Tammuz, Aras, Rabia, Cham 4 and Cham 6) sown, grown and harvested in the same location. The origin of Tammuz, Aras and Rabia is Iraq while the origin of Cham 4 and Cham 6 is Syria (ICARDA). Costantino was used as a reference in this study. Cham 4 and Cham 6 were introduced to Iraq by FAO (Food and Agriculture Organization) since 1997.

2.2. SDS-PAGE Electrophoresis

2.2.1. HMW-GS Extraction

The grain protein HMW-GS was analyzed by using SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis). The grains (30 gram) were ground to fine powder and 20 mg was weighed in 1.5 ml microtube. Three hundred microliter of protein extraction buffer [28.5% sample buffer (7% SDS, Tris-HCl 0.01 M (pH 6.8), 30% glycerol, 0.001% Coomassie bleu), 5% 2-mercaptoethanol] was added to each micro tube, kept 2 hr at room temperature (27°C) and centrifuged at 13000 rpm for 10 min. The supernatant contains dissolved extracted protein HMW-GS ready for experiment purposes, which could be kept for longer time at 4°C. Before the loading of samples on the SDS-PAGE gel, the samples were heated at 80°C for 20 min and then loaded on SDS-PAGE. The gel consisted of a 15% separating gel (pH 8.4), beneath a 3% stacking gel (pH 6.8). Electrophoresis was carried out at room temperature using a home-made vertical electrophoresis apparatus, and the running was performed at 15 mA/gel for 18 hours. After 18 hours, the gels were stained in 12.5% (w/v) trichloroacetic acid, 0.01% (w/v) Coomassie Brilliant Blue R250 and distained with distilled water (Akhtar et al., 1994).

2.2.2. LMW-GS Extraction

The flour (20 mg) was added to 500 µl 50% (v:v) propan-2-ol at 60°C for 30 min with agitation every 10 min at room temperature, then centrifuged at 13000 rpm for 10 min. The residue was washed with 500 µl 50% propan-2-ol for 30 min at 60 ºC. After centrifuging at 13000 rpm for 10 min, the supernatant was again discarded. This step to remove gliadins was repeated three times. Glutenin was then solubilized with 500 µl of solution [50% (v:v) propan-2-ol, 0.08 M Tris-Hcl (pH 8.5), 20 mM dithiothretol] at 60°C for 30 min. The supernatant was diluted with 1 volume of solution [50% (v:v) propan-2-ol, 0.08 M Tris-HCl (pH 8.5), 40 mM 4-vinylpyridine], and incubated for 3 hr at 60°C. Glutenin was precipitated by 1 ml acetone and the dried pellet was solubilized in 200 µl of buffer [7% SDS, Tris-HCl 0.01 M (pH 6.8), 30% glycerol, 0.02% Bromophenol blue or 0.001% Coomassie bleu]. Finally, 30 µl samples were loaded into the slots of SDS-PAGE (Cherdouh et al., 2005). The gel consisted of a 15% separating gel (pH 8.4), beneath a 3% stacking gel (pH 6.8). Electrophoresis was carried out at room temperature using a home-made vertical electrophoresis apparatus, and the running was performed at15 mA/gel for 18 hours. After 18 hours, the gels were stained in 12.5% (w/v) trichloroacetic acid, 0.01% (w/v) Coomassie Brilliant Blue R250 and distained with distilled water (Akhtar et al., 1994).

2.2.3. Two Dimensional Gels A-PAGE X SDS-PAGE

The two dimensional Acid-PAGE x SDS-PAGE was performed by the protocol as described by Pagne et al., 1984. After the first dimension A-PAGE (Acid

polyacrylamide gel electrophoresis), the gels were cut into single strips and incubated for 15 min in 0.0625 M Tris-HCl (pH 6.8), 2% (w/v) SDS, 40% (w/v) glycerol. The strips were then loaded onto a SDS-PAGE gel prepared as described above. Gels were run at 40mA/gels at room temperature and stopped 30 min after the tracking dye had reached the bottom of the gel. They were stained as described above.

2.3. Quantification of Protein

Percentage of nitrogen was determined on 0.25 g of flour by the Dumas combustion method using a nitrogen analyzer according to Approved Method 38-12 (AACC 2000) and reported as protein by N*6.25. (American Association of Cereal Chemists. 2000 approved method of the AACC, 9th ed. Method 38-12).

2.4. SDS-Test

Five ml of distilled water put in the cylinder and 0.5 g of flour added to the cylinder. The cylinder was closed and shaken 15 times (one per second) at the first minute and at the second minute; the cylinder was shaken again 15 times (one per second). At the completion of 3 min and 45 second, the cylinder again was shaken 15 times (one per second) and 5 ml of SDS/ lactic acid [20 g of SDS in 1L of distilled water and 20 ml of mix (10 ml lactic acid 88% and 80 ml of distilled water) was added to SDS solution] added to cylinder and the cylinder was shaken 4 times. At the completion 6, 8 and 10 minute, the cylinder was shaken again 4 times. The volume of sediment read at the completion 25 min. The value obtained is multiplied by 10 to obtain a value of sedimentation volume compared with 100ml of solution.

2.5. Measurement of Quality Traits

- Thousand Kernel Weight: We prepared a 300 gram of wheat grain by removing all dockage, shrunken and broken seeds, and other foreign material. The samples (300 gram /variety) divided into five lots of 60 gram and the number of seeds was calculated for each lot.
- Length And Large Kernel Diameter: the diameter of kernel was calculated by taking several photos of five lots of each variety (10 gram/lot). The diameter was determinate by Image-J software.
- Flour Yield: five lots of 60 gram of each variety were weighted before and after grinding. The flour yield was measured by the difference between the weight of kernel before grinding and the weight of flour yield.

2.6. Data Analysis

Electrophoregrams for each variety were scored and the presence (1) or absence (0) of each band noted. Presence and absence of bands were entered in a binary data matrix. LSD (least significant difference) test was carried out using a statistical package SPSS-PC, version 15. UPGM (Unweighted Pair Group Method with Arithmetic) used for construct the dendrogram.

3. Results and Discussion

3.1. Characterization of Wheat Varieties by Quality Evaluation

Thousand kernel weight, length diameter kernel, larger diameter kernel, protein content, SDS-Test and SDS index showed significant differences, while flour yield was not significant (Table 1). Costantino gave the lowest thousand kernel weight and Aras gave the highest value. In terms of kernel quality, inverse relationships have been reported between the kernel size and protein content (O'Brien and Ronalds, 1984). The highest value for length kernel diameter was that of Tammuz, Aras and Rabia while Cham 4, Costantino and Cham 6 showed lower value for length kernel diameter (Table 1). The varieties Cham 4, Cham 6 and Tammuz had the highest values of larger kernel diameter and Aras, Costantino and Rabia had the lowest values. The flour protein content ranged from 8.14 to 9.24 and Costantino showed the lowest value. According to the SDS-test, the varieties were divided into five groups. The SDS-test of Tammuz was highly significant than the rest of varieties. The variety Cham 4 had the lowest value of SDS-test suggesting poor insoluble protein content. The SDS-sedimentation volume correlated with the amount of total HMWG subunits and individual HMWG subunits. Some subunits were positively correlated, and the others were negatively correlated with the sedimentation volume (Seilmeier et al., 1991). Carrillo et al. (1990) reported that HMWG subunits had additives and epistatic effects on the SDS-sedimentation volume. Rharrabti et al. (2003) studied the relationship between some quality traits and yield of durum under different conditions.

3.2. Characterization of Wheat Varieties by SDS-PAGE

3.2.1. Allelic Variation of HMW-GS Subunits at Glu-1

The wheat varieties analyzed showed four different HMW glutenin banding patterns (Table 2). The frequency of occurrence of HMW glutenin subunits with composition of 1 (50%), 7+9 (16.67%), 7 (50%), 20 (33.33%), 5+10 (33.33%) and 2+10 (66.67%). Subunit 20 had different effects on the functional properties in three subunits at the Glu-D1 allele. Subunit 20 with a background of subunits 2+12 had a smaller negative effect than subunits 5+10 and subunits 2.2+12 (Kanenori et al., 2003). Subunits 17+18 had higher values of functional properties than subunits 7+9 with a background of subunits 2.2+12 (Kanenori et al., 2003). On the other hand, based on the analysis of various cultivars from several countries, Moonen et al., (1983) reported that the Glu-A1b alleles exerted stronger effects on the SDS sedimentation than Glu-A1a.

The varieties Tammuz, Aras and Costantino showed the presence of allele 1 at the locus Glu-A1. On the other hand, the varieties Rabia, Cham 4 and Cham 6 revealed the absence of allele at the locus Glu-A1 (Table 2). All varieties showed the presence of alleles at locus Glu-B1. At Glu-B1, there are three types of alleles a, c, e (7, 7+9 and 20) (Tables 2 and 3). The SDS-PAGE analysis showed two types of alleles at locus Glu-D1: a, d (5+10 and 2+12) (Tables 2 and 3). Figure 2 showed the two dimensional A-PAGE x SDS-PAGE maps of reduced and alkylated glutenin from wheat varieties. The HMW-GS of glutenin were identified easily (Figure 2). The results confirmed the significantly beneficial effects of the Glu-D1d on the dough and gluten strength (Tadashi et al., 2006). Kanenori et al. (2003) showed that the Glu-A1a and Glu-A1b alleles exerted similar effects on the gluten score.

Table 1. Thousand kernel weight, length kernel diameter, large kernel diameter, flour yield, flour protein content, SDS-sedimentation volume and SDS index of wheat varieties.

Varieties	TKW (g)	LEKD (mm)	LAKD (mm)	FY (%)	PC (%)	SDS-test (ml)	SDSi
Tammuz	40.80 ab	6.25 a	3.14 a	74.82 a	9.15 a	40 a	4.37 a
Aras	41.82 a	6.17 a	2.47 b	74.07 a	9.09 a	35 b	3.85 b
Rabia	35.62 cd	6.11 a	2.07 c	73.64 a	9.03 a	22 e	2.43 d
Costantino	34.61 d	5.52 b	2.14 c	74.92 a	8.14 b	30 c	3.68 b
Cham 4	36.34 c	5.54 b	3.19 a	74.36 a	9.24 a	24 d	2.59 cd
Cham 6	39.67 b	5.40 b	3.16 a	74.52 a	9.09 a	25 d	2.75 c
LSD	1.32	0.33	0.25	1.87	0.35	2.72	0.28

TKW: Thousand kernel weight, LESD: Length kernel diameter, LASD: Large kernel diameter, FY: Flour yield, PC: Protein content, SDS-test: Sedimentation Test, SDSi: Sedimentation Index.Values shown by the same letter are not significantly different (p=0.05) by LSD test.

Table 2. HMW subunits composition and quality score of wheat varieties.

Varieties	Glu:A1	Glu:B1	Glu:D1	Quality Score
Tammuz	1	7+9	5+10	14
Aras	1	20	5+10	10
Rabia	Null	20	2+12	5
Costantino	1	7	2+12	7
Cham 4	Null	20	2+12	5
Cham 6	Null	20	2+12	5

Table 3. Allele's composition at the Glu-1 loci of bread wheat varieties.

Varieties	Glu:A1	Glu:B1	Glu:D1
Tammuz	a	c	d
Aras	a	e	d
Rabia	-	e	a
Costantino	a	a	a
Cham 4	-	a	a
Cham 6	-	a	a

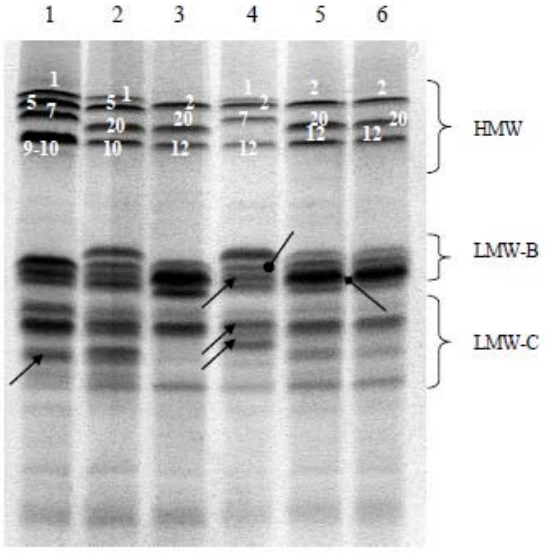

Figure 1. SDS-PAGE gel showing the HMW and LMW-GS glutenins of bread wheat varieties.
1: Tammuz, 2: Aras, 3: Rabia, 4: Costantino, 5: Cham 4, 6: Cham 6. Arrow head: alleles encoded by locus Glu-D3, arrow square: alleles encoded by Gl-B3, arrow circle: alleles encoded by Glu-A3.

From the electrophoretic spectra the individual HMW glutenin subunits were determined and so Glu-quality score was calculated (Table 2). The highest value of Glu-score was achieved by the variety Tammuz. On the other hand, Cham 4 and Cham 6 showed the lowest value of score quality (Table 2). These results show that the variety Tammuz contains more of insoluble protein than others of varieties. The couple of subunits 5+10 is the good marker for breading-making quality and the subunit 20 is the marker for weak wheat quality (Payne and Lawrence 1983, Payne et al., 1987a). HMW-GS may be used as a molecular marker of bread-making quality of wheat. The verified correlations between bread-making quality and specific HMW subunit of glutenin can be utilized by wheat breeders using SDS-PAGE of proteins as a screening test for the prediction of breading-making quality of wheat.

Several electrophoretic techniques have been applied to separate the subunits of glutenin in bread wheat cultivars. The method proposed by Gupta and Shepherd (1990) consists of a two-step, one-dimensional fractionation by SDS-PAGE in which glutenin is reduced before being loaded onto a gradient gel for the second step. A great number of bread wheat cultivars have been analyzed by this method. Three different groups of electrophoretic patterns were identified and attributed to genomes A, B and D. A similar approach was proposed by Khelifi and Branlard (1992) and Redealli et al., (1995). Redealli (1995) showed the fraction of HMW-GS and LMW-GS of glutenin by one and two dimensional gels. The storage proteins of hexaploid wheat are important nutritionally but, above all, because of the unique cohesive-elastic properties they bestow on dough made from wheat flours. The HMW subunits of glutenin are considered to be the most important components with respect to the baking quality. Correlations have been established between particular HMW glutenin subunits and bread-making quality of wheat (Payne et al., 1987a; Kriac et al., 1997).

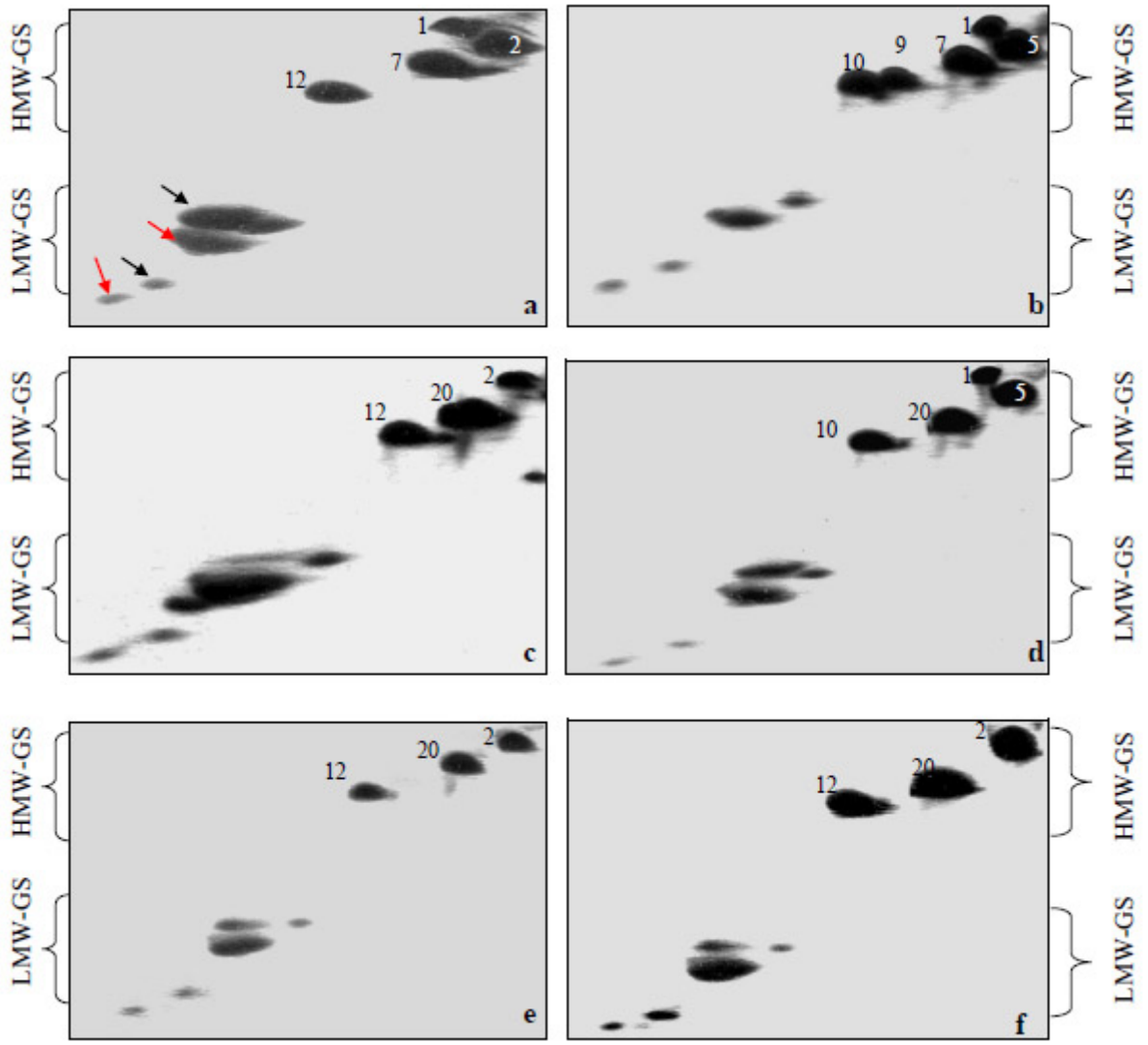

Figure 2. Two-dimensional A-PAGE x SDS-PAGE fractionation of HMW and LMW-GS from bread wheat varieties. a: Costantino, b: Tammuz, c: Rabia, d: Aras, e: Cham 6, f: Cham 4.

3.2.2. Allelic Variation of LMW-GS Subunits at Glu-3

Low molecular weight glutenins are important because the variation in LMW-B glutenin decides the end use quality. We studied the LMW-GS composition of the six wheat varieties by SDS-PAGE analysis. SDS-PAGE profiles of LMW glutenins showed five different LMW glutenin subunits at Glu-3 loci (Figure 1, Table 4). The differences between the patterns were for mobility of bands. The LMW-5 is the most frequent type and the rest of them are present only in one variety. We detected the B and C subunit of LMW-GS in all varieties (Figure 1). The SDS-PAGE gel also showed the C subunit of LMW-GS with a higher mobility than B subunit of LMW-GS (Figure 1). Each variety had 5-6 bands of LMW glutenin B and C subunits coded Glu-A3, Glu-B3 and Glu-D3 loci (Table 4). The same variations were detected in the LMW-Gs among the varieties. According to the profile of LMW subunit B, the varieties divided into four groups: group 1 contains Tammuz, group 2 includes Rabia, group 3 contains Aras and Costantino, group 4 includes Cham 4 and Cham 6. On the other hand, the profile of LMW subunit C divides the varieties into five groups: group 1= Tammuz, group 2= Aras, group 3= Rabia, group 4= Costantino, group 5= Cham 4 and Cham 6. Several LMW subunits in the SDS-PAGE patterns are not attributed to any specific Glu-3 locus because of the overlapping between polypeptides encoded by different alleles (Pogna et al., 1995). Low

Table 4. Allele's composition at the Gli-3 loci and LMW pattern of bread wheat varieties.

Varieties	Glu:A3	Glu:B3	Glu:D3	LMW pattern
Tammuz	a	m	k	LMW-1
Aras	a	m	k	LMW-1
Rabia	a	d	j	LMW-3
Costantino	a	m	k	LMW-4
Cham 4	a	d	j	LMW-5
Cham 6	a	d	j	LMW-5

molecular glutenin subunits are important in determining the dough viscoelastic properties of hexaploid and tetraploid wheat flours (Pogna et al., 1990). However, the basis of differences in effects of different low molecular weight subunits alleles on dough properties is still largely unknown. Gupta and MacRitchie (1991) have shown that the Glu-A3m produces no major B subunit, whereas allele Glu-A3a codes for the major B subunit. This allelic difference was found to be responsible for variation in both the size distribution of the glutenin polymers and dough strength.

3.3. Genetic Diversity among The Varieties

In this study SDS-PAGE of grain storage proteins HMW and LMW-GS was performed in order to analyze molecular weight of gluten subunits and investigate genetic diversity among different wheat varieties. The electrophorogram showing proteins banding pattern of different wheat varieties are given in Figures 1. A total of 18 bands were obtained among which 12 bands were show variation but the other bands common in all varieties (Figure 1).

To investigate evolutionary relationships among the bread wheat varieties according to HMW and LMW glutenin subunits, phylogenetic trees were drawn from the alignment of these varieties based on both HMW and LMW-GS (Figure 3). Cluster analysis of wheat grain storage proteins was performed on the results of SDS-PAGE using the software UPGMA to find out the diversity among the given wheat varieties. The Alignment indicated that the phylogenetic tree was divided into three parts: group 1= Cham 4, Cham 6, Rabia; group 2= Tammuz, Aras and group 3= Costantino. The varieties Cham 4 and Cham 6 showed more similarity than others varieties. On the other hand, Costantino showed more distance with the others varieties. Genetic diversity of European spelts wheat was evaluated by constructing the dendrogram for HMW and LMW glutenin subunit bands (Xueli et al., 2005). Fufa et al. (2005) reported that the genetic diversity estimates based on seed storage protein were lowest because they were the major determinants of end-use quality, which is a highly selected trait.

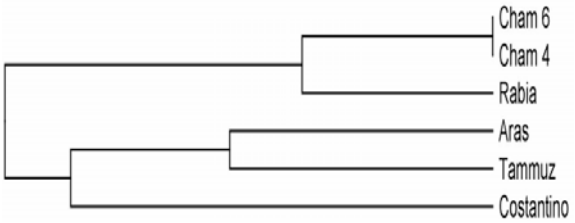

Figure 3. Dendrogram of bread wheat varieties showing the relationship among the varieties based on SDS-PAGE-HMW and LMW-GS.

In conclusion, the results showed that there was the significant difference among the varieties for some of quality traits tested. The electrophorogram revealed that HMW subunit 7+9 which related with the strong quality of wheat for baking is less frequent in the varieties tested. In contrast, HMW subunits 2+12 and 20 which related with the weak quality for making of bread is more frequent in the varieties tested. The LMW-B subunits revealed more high level of polymorphisms than LMW-C subunits. Identification of glutenin subunits in bread wheat varieties in Iraq may be useful for selection aims in breeding programs to determine the relationship between gluten visco-elastic properties.

Acknowledgements

The author thanks the agriculture department of Sulaimanyh for providing the seeds and LNCV and Unità di ricerca per la maiscoltura,Via Stezzano, 24, Bergamo, Italy especially Dr. Rita REDAELLI for help and supporting the work

References

Akhtar H and Odean ML.1994. Characterization of the 1B/1R translocation in wheat using water extractable protein concentrate. Euphytica. 78: 109-113.

American Association of Cereal Chemists. 2000. Approved methods of the AACC, 10th ed. Method 38–12A Wet gluten, dry gluten, water-binding capacity, and gluten index. The American Association of Cereal Chemists, Inc., St. Paul, MN, USA.

Carrillo JM, Rousset M, Qualset CO & Kasadora DD. 1990. Use of recombinant inbred lines of wheat for study of associations of high-molecular-weight glutenin subunit alleles to quantitative traits 1. Grain yield and quality prediction tests. Theor. Appl. Genet. 79: 321–330.

Cherdouh A, Khelifi D, Carrillo JM, Nieto-Taladriz MT. 2005. The high and low molecular weight glutenin subunit polymorphism of Algerian durum wheat landraces and old cultivars. Plant breeding. 4: 338 – 342.

Ciaffi M, Margiotta B, Colaprico G, De-Stafani E, Sgrulletta D, and Lafiandra D. 1995. Effect of high temperatures during grain filling on the amount of insoluble proteins in durum wheat. J. Cereal Sci. 49: 285-296.

Fufa H, Baenziger PS, Beecher I, Dweikat V, Graybosch RA, Eskridge KM. 2005. Comparison of phenotypic and molecular marker-based classifications of hard red winter wheat cultivars. Euphytica. 145: 133-146.

Greenfield JJA, Ross-Murphy SB, Tamas L, Bekes F, Halford NG, Tatham AS & Shwery PR. 1998. Rheological properties of monomeric and polymeric forms of C-hordein, sulphur-poor prolamins of barley. J. Cereal Sci. 27: 233-236.

Gupta RB & Shepherd KW. 1990. Two-step one dimensional SDS-PAGE analysis of LMW subunits of glutelin. Variation and genetic control of the subunits in hexaploid wheat. Theor. Appl. Genet. 80: 65–74.

Gupta RB, Bekes F & Wrigley CW. 1991. Prediction of physical dough properties from glutenin subunit composition in bread wheats. Cereal Chem. 68: 328–333.

Gupta RB and MacRitchie F. 1991. A rapid on-step one dimensional SDS-PAGE procedure for analysis of subunit composition of glutenin in wheat. J. Cereal Sci. 14: 105-109.

Gupta RB, Paul GB, Cornish GA, Palmer FB & Rathjen AJ. 1994. Allelic variation at glutenin subunit and gliadin loci, *Glu-1*, *Glu-3* and *Gli-1*, of common wheats. 1. Its additive and interaction effects on dough properties. J. Cereal Sci. 19: 9–17.

Gupta RB, MacRitchie F. 1994. Allelic variation at glutenin subunit and gliadin loci, *Glu-1, Glu-3* and *Gli-1* of common wheats. II. Biochemical basis of the allelic effects on dough properties. J. Cereal Sci. 19 :19–29.

Kanenori T, Zenta N, Wakako F, Tatsuo K & Hiroaki Y. 2003. Difference in Combination between *Glu-B1* and *Glu-D1* Alleles in

Bread-Making Quality Using Near-Isogenic Lines. Food Sci. Technol. Res. 9 (1): 67–72.

Karic J, Gregova E, Tisova V. 1997. Genetic variability at the Glu-1 loci in old and modern wheats (*Triticum aestivum* L.) cultivated in Slovakia. Genet. Res. Crop Evol. 44: 301–306.

Khelifi D & Branlard G. 1992. The effects of HMW and LMW subunits of glutenin and of gliadins on the technological quality of progeny from four crosses between poor bread making quality and strong wheat cultivars. J. Cereal Sci .16: 195–209.

Lafiandra D, Masci S, Margiotta B & De Ambrogio E. 1998. Development of durum and bread wheat with increased number of High Molecular Weight glutenin subunits. Pages 261-264. in. Proc 9th International Wheat Genetics Symposium, Vol. 4. Grain Quality. A.E. Slinkard. ed. University of Saskatchewan: Saskatoon, Canada.

Lawerence GJ, MacRitchie F & Wrigley CW. 1998. Dough and baking quality of wheat lines deficient in glutenin subunits controlled by Glu-A1, Glu-B1 and Glu-D1 loci. J. Cereal Sci. **7**: 109-112.

MacRitchie F and Lafiandra D. 2001. The use of near-isogenic wheat lines to determine protein composition-functionality relationship. Cereal Chem. 78: 501-506.

Masci S, D'Ovidio R, Lafiandra D & Kasarda DD. 1998. Characterization of a low-molecular-weight glutenin subunit gene from bread wheat and the corresponding protein that represents a major subunit of the glutenin polymer. Plant Physiol. 118:1147-1158.

Moonen JHE, Scheepstra A & Graveland A. 1983. The positive effects of the high molecular weight subunits 3 + 10 and 2* of glutenin on the bread making quality of wheat cultivars. Euphytica. 32: 735–742.

Nagamine T, Kai Y, Takayama T, Yanagisawa T. & Taya. 2000. Allelic variations of glutenin subunit loci Glu-1 and Glu-3 in southern Japanese wheat and their effects on dough and gluten properties. Cereal Sci. 32: 123–135.

O'Brien L & Ronalds JA. 1984. Yield and quality interrelationships amongst random F_3 lines and their implications for wheat breeding. Australa. J. of Agri. Res. 35(4): 443 – 451.

Payne PI, Law CN, Mudd EE. 1980. Control by homoeologous Group 1 chromosome of the high-molecular-weight subunits of glutenin, a major protein of wheat endosperm. Theor. Appl. Genet. 58: 113-120.

Payne PI & Lawerence GJ. 1983. Catalogue of alleles for the complex gene loci, Glu-A1, Glu-B1, Glu-D1 which code for high-molecular-weight subunits of glutenin in hexaploid wheat. Cereal Res. Comm. 11: 29-35.

Payne PI, Jackson EA, Holt LM, Law CN. 1984. Wheat storage proteins: Their genetics and their potential for manipulation by plant breeding. Philos. T. Roy Soc B. 304:359-371.

Payne PI, Nightingale MA, Krattiger AF & Holt LM. 1987a. The relationship between HMW gutenin subunit composition and the bread-making quality of British-grown wheat varieties. J. Sci. Food Agr. 40: 51–65.

Payne PI, Seekings JA, Worland MG, Jarvis M, Holt L. 1987b. Allelic variations of glutenin subunits and gliadins and its effect on breadmaking quality in wheat: Analysis of F5 progeny from Chinese Spring X Chinese Spring (Hope 1A). J. Cereal Sci. 6: 103--118.

Pogna NE, Redaelli R, Vaccino P, Biancardi AM & Peruffo ADB. 1995. Production and genetic characterisation of near-isogenic lines in the bread wheat cultivar Alpe. Theor Appl. Genet. 90:650-658.

Pogna PE, Autran JC, Mellini F, Lafiandra D & Feillet P.1990. Chromosome 1B-encoded gliadins and glutenin subunits in durum wheat: genetics and relationship to glutenin strength. J. Cereal Sci. 11: 15–34.

Redaelli R, Morel MH, Autran JC & Pogna NE.1995. Genetic analysis of low M_r glutenin subunits fractionated by two-dimensional electrophoresis (A-PAGE × SDS-PAGE). J. Cereal Sci. 21:5-13.

Rharrabti Y, Villegas D, Royo C, Martos-Núñez V & García del Moral LF. 2003. Durum wheat quality in Mediterranean environments: II. Influence of climatic variables and relationships between quality parametersField. Crops Res. 133-140.

Rogers WJ, Miller TE, Payne PI, Seekings JA, Holt LM & Law CN. 1997. Introduction to bread wheat (*Triticum aestivum* L.) and assessment for bread making quality of alleles from T. boeticum Boiss. Ssp. Thaoudar at Glu-A1 encoding two high-molecular weight subunits of glutenin. Euphytica. 93: 19-29.

Seilmeier W, Belitz HD. and &Weiser H. 1991. Separation and quantitative determination of high-molecular-weight subunits of glutenin from different wheat varieties and genetic variants of the vari ety Sicco. Z. Lebensm Unters Forsch. 192: 124–129.

Tadashi T, Shojiro I and Tatsuya M. Ikeda. 2006. Effects of High-molecular-weight and Low-molecular-weight Glutenin Subunit Alleles on Common Wheat Flour Quality. Breeding Sci. 56 (2): 131-136.

Xueli A, Qiaoyun L, Yueming Y, Yinghua X, Hsam SLK, Zeller FJ. 2005. Genetic diversity of European spelt wheat (*Triticum aestivum* ssp. *spelta* L. em. Thell.) Revealed by glutenin subunit variations at the *Glu-1* and *Glu-3* loci. Euphytica. 146(3): 193-201.

JJBS

Jordan Journal of Biological Sciences

Volume 2, Number 2, June 2009
ISSN 1995-6673
Pages 63 - 68

The Role of Silymarin in the Protection of Mice Liver Damage Against Microcystin-LR Toxicity

Wahsha M. , Al-Jassabi S.*

Department of Biological Sciences, Yarmouk University, Jordan

Abstract

The presence of cyanobacterial toxins in drinking and recreational waters represents a potential risk to public health. Microcystin-LR (MC-LR) is a potent cyclic heptapeptide hepatotoxin produced by the blue-green algae *Microcystis aeruginosa* (*M. aeruginosa*). Chemoprotectant studies suggest that membrane-active antioxidants may offer a protection against microcystin toxicity. The aim of this study is to investigate the potential benefits of dietary supplementation of silymarin as antioxidant on microcystin toxicity in mouse livers. A group of Balb/c mice was pre-treated for ten days with silymarin (extracted from milk thistle seeds collected from local areas of north Jordan) (400 mg of silymarin /Kg mouse body weight given orally once a day for 10 days), before an intraperitoneal injection (i.p) with 200 µg toxin/kg mouse body weight of MC-LR (according to LD_{50} value). Pre-treatment of mice with a single dose of silymarin, aflavonolignane (Silybum marianum L. Geartin) completely abolished the lethal effects and significantly decreased the levels of serum enzymes, alanine aminotransferase (ALT), lactate dehydrogenase (LDH) and gamma glutamyl transferase (GGT), inhibition of Protein phosphatase (PP1) , level of methylglyoxal (MG) and lipid peroxidation (LPO) as MDA amount induced by MC-LR. Therefore, silymarin supplied as dietary supplement may have protective effects against chronic exposure to MC-LR.

الملخص

يشكل وجود سموم الطحالب الزرقاء الخضراء (السيانوبكتريا) في مياه الشرب والري مشكلة صحية خطيرة للغاية . يمتاز هذا النوع من السموم بانه ذا سمية عالية الكفاءة وخاصة لنسيج الكبد خيث وجد انه يسبب تلف نسيج الكبد بسرعة فائقة اضافة الى ما يسببة من اورام سرطانية . وهذا النوع من السموم الطحلبية هو عبارة عن ببتيد سباعي حلقي يقوم بتكوينه نوع من الطحالب الزرقاء -الخضراء والمسماة بالمايكروسستس اريجينوزا . وقد اثبتت الدراسات الخاصة باستخدام الغذائية التي تساعد في حماية الانسجة الحية بان مضادات الاكسدة قد تساعد في الحماية من هذا النوع من السموم . ان هدف هذه الدراسة هو البحث عن امكانية استخدام مضادات الاكسدة الغذائية كالسليمارين (المستخلص من نبات حليب مريم الموجود في المناطق الشمالية من المملكة) في حماية كبد الفار من التلف الذي تسببه له هذه السموم . لقد تم اخذ مجموعة من الفئران من نوع Balb/c وتم اطعمها بمستخص السليمارين بمقدار 400 ملغم لكل كغم من جسم الفأر ولمدة عشرة ايام (مرة واحدة يوميا ولمرة واحدة) ومن ثم حقنها في اليوم الحادي عشر بسموم المايكروسستين تحت الجلد بجرعة مقدارها 200 مايكروغرام لكل كغم من وزن الفار (وذلك نسبة الى قيمة LD50) التي تم قياسها . ومن ثم تم قياس كفاءة السليمارين عن طريق قياس كمية كل من الانزيمات الالانين ترانسفيريز ، اللاكتيت ديهيدروجينيز والكلوتاميل ترانسفيريز في مصل الدم . وقياس كل من البروتين فوسفاتيز والمثيل كلايوكزاليز ، ومقدار اكسدة الليبيدات (قياس كمية المالون ثنائي الالدهيد في مستخلص نسيج الكبد .وقد وجد في هذه الدراسة ان السليمارين يتمتع بكفاءة عالية كمضاد للاكسدة التي يسببها هذا النوع من السموم وبذلك يوفر الحماية المطلوبة لكبد الفأر من التلف.

Keywords: Chemoprotection; Toxicity; Microcystin; Silymarin.

1. Introduction

The rising eutrophication by human activities of fresh waters, including drinking water reservoirs, has increased the occurrence and intensity of cyanobacterial blooms (Ordorika *et al*. 2004; Antoniou *et al*., 2008). Microcystins (MCs), specific hepatotoxins produced by numerous cyanobacterial species (primarily *M. aeruginosa*) in eutrophic surface waters have risen, increasing worldwide concern (Andrinolo *et al*., 2008; Billam *et al*., 2008). MCs are characterized as monocyclic heptapeptide with over 70 different MCs isoforms identified (Spoof and Meriluoto, 2002). The general structure of microcystin is cyclo (D-Ala-L-X-D-MeAsp-L-Z-Adda-D-Glu-Mdha) where X and Z represent. The two variable amino acids and Adda is 3-amino-9-methoxy- 2, 6, 8-trimethyl-10-phenyl-deca-4, 6-dienoic acid (Ordorika *et al*., 2004; Oriola and Lawton,2005).

Microcystin-LR (MC-LR) is the most widely distributed and studied MCs variant (Gupta and Guha, 2006). It has the amino acids leucine and arginine at positions 2 and 4, respectively (Spoof and Merilluoto, 2002; Antoniou *et al*., 2008). MC-LR has been found to be a potent inhibitor of protein phosphatase type 1 and type 2A (Andrinoloa *et al*.,2008), resulting in the disturbance of many important cellular processes (Oriola and Lawton, 2005). Exposure to MC-LR has been shown

* Corresponding author. sjassabio@hotmail.com.

to cause oxidative stress in various organisms (Leao *et al*., 2008). Formation of reactive oxygen species (ROS) and oxidative stress is associated with the development of many pathological states (Gupta and Guha,2006; Billam *et al*., 2008). Oxidative stress may occur either due to the decrease of cellular antioxidant level, or due to the overproduction of ROS (Jayaraj *et al*.,2006). Exposure to MC-LR has been linked with increase of ROS production not only in domestic and wildlife animals, but also in human (Weng *et al*., 2007).

Recently, interest has increased considerably in finding naturally occurring antioxidants to replace synthetic antioxidants, which are being restricted due to their carcinogenicity (Looa *et al*., 2007). Silymarin was shown to be an antihepatotoxic agent against MC-LR toxicity in cultured rat hepatocytes(Mereish and Solowe, 1990). Mereish et al., (1991) extended their studies to *in vivo* and tested the use of silymarin against MC-LR toxicity in mice and rats, found that this compound acted potentially against it. Rao et al., (2004) concluded that silymarin could offer great protection against MC-LR toxicity.

The goal of this study is to investigate the role of naturally- extracted silymarin (one of the active polyphenol antioxidants in milk thistle seeds) pre-treatment in protection against MC-LR (extracted from King Talal Reservoir, Jordan)-induced liver injury in Balb/c mice.

2. Materials and Methods

2.1. Chemicals

All chemicals used in this study were of analytical grade and were purchased from Sigma Chemical Co. USA.

2.2. Samples

Samples of *M. aeruginosa* cells were collected according to (Al-Jassabi and Khalil, 2006) from selected sites of KTR in Jordan during blooming season (July, August and September, 2007). Microcystin was extracted from the lyophilized cells of *M. aeruginosa* according to Lawton *et al*., (1994), and the LD_{50} of the toxin extract was determined according to Fawell *et al*., (1999), in which LD_{50} is determined as the value between the lowest toxin dose at which mice die and the highest toxin dose at which mice live after administration of a toxin.

2.3. Silymarin Extraction

Milk thistle seeds were collected from specific areas in Jordan (Ajlun & Irbid) during July and August 2007. The silymarin was extracted according to the method reported by Duan *et al*., (2004).

2.4. Animal Treatment and Sample Collection

Male Balb/c mice 6-7 weeks old (average body weight 30 g) were used in this study. Mice were obtained from animal house/ Yarmouk University and were maintained on standard laboratory diet and tab water throughout the experiments. Five animals were maintained in each cage (stainless) under a 12-12h light-dark cycle and room temperatures of 23-26^{o}C.

Sixty mice were used in this study, and they were assigned into 4 groups (10 mice each) as follows: Group 1 was the control group (C), without supplementation of silymarin or treatment with toxin; Group 2 was the toxin control group (with 2 sub groups, 10 mice each) (T_6 and T_{12}), treated with toxin only, intraperitoneal injection (i.p) with 200 µg toxin / kg mouse body weight. 10 mice were killed after 6 h and 10 mice were killed after 12 h; Group 3 was the silymarin control group (SC), supplemented orally with 400 mg silymarin /kg mouse body weight daily for 10 days according to Lakshmana *et al*., (2004), then they were killed; Group 4 (silymarin and toxin) with two sub group (10 mice each) supplemented orally with 400 mg silymarin / kg mouse body weight daily for 10 days, then were injected i.p. with 200 µg toxin/kg mouse body weight. 10 mice were killed after 6 h (ST_6) and 10 mice were killed after 12 h (ST_{12}). Blood was collected immediately after sacrification of mice, and serum was isolated and stored at -20ºC for the biochemical tests. Livers were removed, immediately after death, perfused with normal saline containing heparin, weighed, and homogenized with phosphate buffer saline (pH 7.2) using Ultra Turax homogenizer, centrifuged at 3000g for 30min. The supernatant was removed and stored at -20^{o}C.

2.5. Alanine Aminotransferase (ALT) Assay

Determination of ALT activity in the serum sample was measured according to the procedure recommended by Gehringer *et al*., (2003).

2.6. Cytotoxicity Assay

Serum lactate dehydrogenase (LDH) activity was measured according to Doyle and Griffiths, (1998).

2.7. Gamma Glutamyl Transferase Assay (GGT):

Determination of GGT activity in the serum sample was measured based on Szasz (1974) method .

2.8. Protein Phosphatase (PP1) Assay

Depending on the procedure described by Yuan *et al*., (2006), PP1 activity in liver homogenate was assayed by measuring the rate of formation of the yellow color of p-nitrophenol (*p*-NP) produced by hydrolysis of p-nitro-phenylphosphate (*p*-NPP) in an alkaline solution spectrophoto-metrically.

2.9. Lipid Peroxidation (LPO) Assay

The lipid peroxidation level of the hepatocyte was measured according to the method described by Hosseinzadeh *et al*., (2007).

2.10. Methylglyoxal Assay (MG)

The glyoxalase system breaks down the toxic methylglyoxal, which is formed as a by-product of the triosephosphate isomerase reaction in glycolysis. Methylglyoxal was determined from liver homogenate according to the method described by Ratliff *et al*., (1996).

2.11. Statistical Analysis

All results were expressed as the mean ± S.E.M from ten mice per group. One way analysis of variance (ANOVA) followed by a Tukey test was used to determine the significance of the differences between the groups. Statistical significance was declared when P value was equal to or less than 0.05. The statistical analysis was performed using the Sigma Stat Statistical Software version 3.5.

3. Results

In this study, MC-LR treated mice showed increased liver body mass index ratio due to massive intrahepatic hemorrhage and pooling of blood in the liver, as shown in Table 1. The livers of mice which received silymarin were within the normal value, but this value increased slightly after the exposure to the toxin.

Treatment with MC-LR resulted in a significant increase in levels of ALT activity compared to the saline-treated group (Table 1). The elevated levels of serum ALT were significantly reduced in mice received toxin and silymarin supplementation. An increased ALT value was revealed in sera by folds of 1.5 in ST_6. A further increase was shown as 4.2, folds for ST_{12} compared to controls ($P<0.05$).

As shown in Table 1, MC-LR alone produced after 6 h of exposure nearly three-fold and five-fold after 12 h increases in serum LDH level compared to control mice, indicating severe liver injury. The mice receiving toxin and silymarin supplementation revealed an increased LDH value in sera by folds of 2 in ST_6. A further increase was shown as 2.5 folds for ST_{12} compared to controls ($P<0.05$).

Mice group, which received silymarin only, show levels of GGT which are within the normal value (39±4U/mg). Furthermore MC-LR administration (200mg toxin/kg mouse body weight) of 6 and 12 h increased serum values of GGT about 3.8 and 5.8 fold, respectively, compared with control mice ($P<0.05$), while the increase was dramatically diminished by silymarin pre-treatment, and it could significantly inhibit the increase of GGT induced by MC-LR as shown in Table 1.

Results of spectrophotometric measurements of protein phosphatase activity of liver homogenates for all groups are presented in Table 1. PP1 activity was significantly inhibited in group 2; 0.46% inhibition has occurred for those of 6 h and 72% for those of 12 h when compared with control. Supplementations with silymarin caused a partial protection of PP1 activity against the action of MC-LR, of almost three-fold compared with the levels in toxin control group.

Control mice exhibited normal levels of lipid peroxidation (LPO) measured as amount of MDA it was 0.067µM in hepatocytes homogenate. There was a dramatic increase in MDA level in liver homogenate from toxin-treated groups by 16 fold in case of T_6 and 34 fold in case of T_{12} when compared with those of control mice ($P<0.05$), as reflected in the elevation of TBA values, which appear time-dependent (Table 1). The mice receiving toxin and silymarin supplementation revealed an increased TBA value by 3 folds in ST_6 and further increase shown as 10, fold for ST_{12} in compared to controls.

The MG concentration in the liver homogenates was determined at 6 and 12h after i.p injection of MC-LR and results were compared with controls as shown in Table 1. It was clear that the effect of MC-LR on MG was time dependent; for mice exposed only to the toxin; results were found to be higher at 12 h compared to those at 6 h. However, pre-treated silymarin group showed a significant protection against accumulation of MG in the hepatocytes.

4. Discussion

M. aeruginosa dominates the cyanobacterial communities of KTR during the warmer season (specifically, from June to October, 2007) (AlJassabi, 2004). Our microscopic investigations ensured that the major cyanobacterial species found in the collected samples were *M. aeruginosa* in agreement with our previous study (Al-Jassabi and Khalil, 2006). The phosphatase inhibitory activity proved the bioactivity of the toxin while the spectrophotometric analysis proved that the extracted toxin was MC-LR (Spoof and Meriluoto, 2002 & Oriola and Lawton, 2005).

The present investigation examined the hepatotoxic effects of MC-LR isolated from *M. aeruginosa* of KTR, in Balb/c mice after intraperitoneal route of exposure to the toxin. Besides we investigated the potential hepatoprotective efficacy of silymarin as naturally isolated antioxidant against MC-LR effects. The LD_{50} concentration of MC-LR by i.p route was determined as 200 µg toxin/kg mouse body weight by a modified Fawell's up-and-down method (Fawell *et al*., 1999) which is at variation with some earlier reports (Ding *et al*., 2006 and Lombardo *et al*., 2006). A wide range of LD_{50} values have been reported for MC-LR by administration in mice.

In agreement with previous studies (Dawson, 1998; Weng *et al*., 2007; Xu *et al*., 2007) the results in toxin group indicate that severe liver damage accompanied by marked change in colour and weight can occur by i.p injection of LD_{50} dose. Hepatocellular damage was first noticed by the increase in total liver size, due to intrahepatic haemorrhage and accumulation of fluids caused by the action of MC-LR (Dufour and Clavien, 2005).

Exposure to MC-LR causes a disturbance of cellular iron homeostasis as a result of ferritin inhibition (Chen *et al*., 2005). Mackintosh *et al*., (1990) reported that Protein phosphatases are inhibited with high affinity by MC-LR. Thus, MC-LR completely blocks access to the active centre of the enzyme using Fe^{+3} as a catalyst (Lohse *et al*., 1995), thereby causing hyperphosphorylation of the cell and a massive disruption of a number of important cellular mechanisms (Yang *et al*., 1997). Silymarin in the mice pre-treated group could partially hinder the inhibition of PP1 binding to Fe^{+3} (Kanaze *et a*l., 2005).

One important consequence of excessive free radical production after exposure to MC-LR is the toxin ability to attack many organic molecules, including polyunsaturated fatty acids in the cell membrane (Denisov *et al*., 2003 and Pinho *et al*., 2005), leading to lipid peroxidation. Several studies reported that ROS can initiate lipid peroxidation through the action of hydroxyl radicals (Joshi *et al*., 2005). Our analysis of hepatocytes for lipid peroxidation showed that silymarin could bring a decrease in the formation of lipid peroxidation through their ability to scavenge the hydroxyl radicals. The activity of silymarin as scavenger of free radicals and ROS has been described by others (Mereish et al., 1991; Rao et al., 2004)

Methylglyoxal is considered to be toxic for mammals (Kalapos, 2008). The cytotoxicity associated with the accumulation of MG after exposure to MC-LR is due to the inhibition of glyoxalase I and the depletion of the action of the antioxidant defense system found in the liver

Table 1. Summary of results of the effect of silymarin supplementation on mice receiving single lethal dose of MC-LR

	C	T_6	T_{12}	SC	ST_6	ST_{12}
Livers weight (g)	1.47 ± 0.02	2 ± 0.057	2.74 ± 0.06	1.45 ± 0.02	1.55 ± 0.05	1.87 ± 0.09
ALT (U/L)	574.6±6.95	1649±43	2461±51	533±5	884±6	1202±39
LDH (U/mg)	987 ± 207	3352± 123	4868 ±46	885 ± 2	2057 ± 67	2471 ± 112
GGT (U/mg)	39 ± 4	132 ± 5	226 ± 7	32 ±4	95 ± 3	163 ±3
PP1 (U/mg)	0.583 ± 0.01	.316± 0.01	0.165± 0.002	0.562±0.02	0.414±0.021	0.31±0.007
LPO (μM)	0.067 ± 0.01	1.3 ± 0.04	2.292 ± 0.04	0.013± 0.001	0.212 ± 0.002	0.705 ±0.027
MG (μM)	1.61±0.075	29.03±0.39	41.94±0.797	1.58±0.0411	16.13±0.345	16.94±0.334

(Aljassabi, 2004). The increase in MG levels in the liver caused significant generation of free radicals which might further strengthen the damage and affect the hepatocyte function (Kalapos, 2008). This was noticed in the toxin group which received the toxin only as shown in the results. Silymarin pre-treated group showed a significant decrease in the accumulation of MG.

The antagonistic effect of silymarin could be similar to that of dithioerythritol by stabilizing protein-thiol, which may be important to the structure of liver cell (Mereish et al., 1991). Previous studies and the present investigation show that among the various chemoprotectants that have been screened till date, silymarin is the most effective in preventing the MC-LR-induced lethality in mice.

References

Al-Jassabi S. 2004. Effect of microcystin from Jordan on ion regulation and antioxidant system in the hepatocytes of mice. J. Biol. Sci. 4(4): 547-552.

Al-Jassabi S. 2004. Effect of methylglyoxal on antioxidant enzymes of the liver and spleen of the mice. J. Biol. Sci. 4(5):605-608.

Al-Jassabi S. and Khalil A. M. 2006. Initial report on identification and toxicity of *Microcystis* in King Talal reservoir, Jordan. Lakes & Reserv.: Res. & Manag. 11: 125-9.

Antoniou MG Shoemaker JA Armah CA and Dionysiou DD. 2008. LC/MS/MS structure elucidation of reaction intermediates formed during the TiO2 photocatalysis of microcystin-LR. Toxicon 3142: 1-40 .

Andrinoloa D Sedanb D Teleseb L Aurab C Maserab S Giannuzzia L Marrac CA and Alaniz MJT. 2008. Hepatic recovery after damage produced by sub-chronic intoxication with the cyanotoxin microcystin LR. Toxicon 51: 457–67 .

Billam M Mukhi S Tang L Gao W and Wang J. 2008. Toxic response indicators of microcystin-LR in F344 rats following a single dose treatment. Toxicon 3139: 3-4 .

Chen T Wang Q Cui J Yang W Shi Q Hua Z Ji J and Shen P. 2005. Induction of apoptosis in mouse liver by microcystin-LR. Mol. & Cell. Proteo. 4(7): 958-74.

Dawson RM. 1998. The toxicology of microcystins. Toxicon 36 (7): 953-62.

Denisov ET , Denisova TG and Pokidova TS. 2003. Handbook of free radical initiators, John Wiley & Sons, Inc, Hoboken, New Jersey, USA, 22-57.

Ding XS Li XY Duan HY Chung IK and Lee JA. 2006. Toxic effects of Microcystis cell extracts on the reproductive system of male mice. Toxicon 48: 973–9.

Doyle A and Griffiths JB. 1998. Cell and tissue culture: laboratory procedures in biotechnology. John Wiley & sons, Baffins Lane Chichester, West Sussex P019 1UD, England, 71-4.

Duan L Carrier DJ. and Clausen EC. 2004. Silymarin extraction from milk thistle using hot water Spring. App. Biochem. and Biotech. 59(6): 113-6.

Dufour JF and Clavien PA. 2005. Signaling Pathways in Liver Diseases, Springer-Verlag, Berlin, Heidelberg, Germany, 47-59.

Fawell JK Mitchell RE Everett DJ and Hill RE. 1999. The toxicity of cyanobacterial toxins in the mouse: I Microcystin-LR. Human & Experimental Toxicol. 18(3): 162-7.

Gehringer MM Downs KS Downing TG Naude RJ and Shephard EG. 2003. An investigation into the effect of selenium supplementation on microcystin hepatotoxicity. Toxicon 41: 1-8.

Gupta US Guha S. 2006. Microcystin toxicity in a freshwater fish, Heteropneustes fossilis (Bloch). Current Science 91(9): 1261-71.

Hosseinzadeh H Parvardeh S Asl MN Sadeghnia HR and Ziaee T. 2007. Effect of thymoquinone and nigella sativa seeds oil on lipid peroxidation level during global cerebral ischemia-reperfusion injury in rat hippocampus. Phytomed. 14: 621–7.

Jayaraj R., Anand T. & Lakshmana Rao PV. 2006. Activity and gene expression profile of certain antioxidant enzymes to microcystin-LR induced oxidative stress in mice. Toxicol. 220(2,3): 136-46 .

Joshi G., Sultana R., Tangpong J., Cole MP., Clair D., Vore M., Estus S. and Butterfield DA. 2005. Free radical mediated oxidative stress and toxic side effects in brain induced by the anti cancer drug adriamycin: Insight into chemobrain. Free Rad. Res. 39(11): 1147–54.

Kalapos MP. 2008. The tandem of free radicals and methylglyoxal. Chemico Biol. Inter. 171(3): 251-271.

Kanaze FI., Gabrieli C., Kokkalou E., Georgarakis M., Niopas I. 2005. Simultaneous reversed-phase high-performance liquid chromatographic method for the determination of diosmin hesperidin and naringin in different citrus fruit juices and pharmaceutical formulations. J. Pharm. Biomed Analysis 33: 243-9.

Lakshmana Rao PV, Gupta N. and Jayaraj R. 2004. Screening of certain chemoprotectants against cyclic peptide toxin microcystin-LR. Indian J. Pharmacol. 36(2): 87-92.

Lawton, LA Edwards C and Codd GA. 1994. Extraction and high performance liquid chromatographic method for the determination of microcystins in raw and treated waters. Analyst 119: 1525-1530.

Leão JC Geracitano LA Monserrat JM Amado LL and Yunes JS. 2008. Microcystininduced oxidative stress in Laeonereis acuta (Polychaeta, Nereididae). Marine Environ. Res.3192: 1-10 .

Lohse DL Denu JM and Dixon JE. 1995. Insights derived from the structures of the Ser/Thr phosphatases calcineurin & protein phosphatase. Structure 3(10): 987-90.

Lombardo M Pinto FC Vieira JM Honda RY Carvalho LR & Kiyota S. (2006) Isolation and structural characterization of microcystin - LR and three minor oligopeptides simultaneously produced by Radiocystis feernandoi (Chroococcales, yanobacteriae): A Brazilian toxic cyanobacterium. Toxicon 47 560–66.

Looa AY Jaina K and Darahb I. 2007. Antioxidant and radical scavenging activities of the pyroligneous acid from a mangrove plant, Rhizophora apiculata . Food Chem. 104(1): 300-7.

Mackintosh C Beattie KA Klumpp S Cohen P and Codd GA. 1990. Cyanobacterial microcystin-LR is a potent and specific inhibitor of protein phosphatases 1 and 2a from both mammals and higher plants. Federation of the European Biochemical Society Lett. 264: 187–92.

Mereish KA Bunner DL Ragland DR and Creasia DA. 1991. Protection against Microcystin-LR-induced hepatotoxicity by silymarin: Biochemistry, histopathology, and lethality. Pharma. Res. 8(2): 273-277.

Oriola GA and Lawton LA. 2005. Detection & quantification of toxins of Microcystis aeruginosa (PCC 7820) by HPLC & protein phosphatase inhibition assayffectof blending various collectors at bulk. Afri. J Sci. Technol. 6(1): 1-10 .

Pinho GL Rosa CM Maciel FE Binachini A Yunes JS Proenca LA and Monserrat JM. 2005. Antioxidant responses and oxidative stress after microcystin exposure in the hepatopancreas of an estuarine crab species. Ecotoxicol. Environ. Safety 61: 353-60.

Rao PVL Jayaraj R and Bhaskar ASB. 2004. Protective efficacy and the recovery profile of certain chemoprotectants against lethal poisoning by microcystin-LR in mice. Toxicon 44: 723-730.

Ratliff DM Vander Jagt DJ Eaton RP and Vander Jagt DL. 1996. Increased levels of methylglyoxal-metabolizing enzymes in mononuclear and polymorphonuclear cells from insulin-dependent diabetic patients with diabetic complications: aldose reductase, glyoxalase I and Glyoxalase II. J Clin. Endocrinol. Metabol. 81(2): 488-92.

Spoof L and Meriluoto J. 2002. Rapid separation of microcystins and nodularin using a monolithic silica C18 column. J Chromat. A 947: 237–45 .

Szasz G. (1974) Methods of Enzymatic Analysi. Natural Toxins 2: 714-715.

Weng D Lu Y Wei Y Liu Y and Shen P. 2007. The role of ROS in microcystin-LR- induced hepatocyte apoptosis & liver injury in mice. Toxicol. 232: 15-23

Xu C. Shu W Qiu Z Chen J Zhao Q. and Cao J. 2007. Protective effects of green tea polyphenols against subacute hepatotoxicity induced by microcystin-LR in mice. Environ. Toxicol. Pharma. 24: 140-8.

Yang J Yoneda K Morita E Imamura S Nam K Lee E and Steinert P. 1997. An alanine to proline mutation in the 1a rod domain of the keratin 10 chain in epidermolytic hyperkeratosis. J. Inves. Dermatol. 109:692–4.

Yuan M Carmichael WW and Hilborn ED. 2006. Microcystin analysis in human sera and liver from human fatalities in Caruaru, Brazil. Toxicon 48: 627–40.

JJBS
Jordan Journal of Biological Sciences

Volume 2, Number 2, June 2009
ISSN 1995-6673
Pages 69 - 76

Effects of Trans-Resveratrol, Isolated from *Smilax Aspera*, on Smooth Muscle, Blood Pressure, and Inflammation in Rats and Nociception in Mice

Abeer H. Harb[a], Musa abu Zarga[b], Shtaywy Abdalla[a,*]

[a]*Deptartment of Biological Sciences* ,[b]*Dept. of Chemistry, University of Jordan, Amman 11942, Jordan*

Abstract

Smilax aspera (Liliaceae) has been used in herbal medicine for the treatment of many disorders. Chemical analysis of this plant resulted in the isolation of the stilbene trans-resveratrol. The effects of this compound on rat isolated ileum and urinary bladder, blood pressure and inflammation, and nociception in mice have been examined. Resveratrol ($3x10^{-5}$ -$1x10^{-1}$ mg/ml; or $1.3x10^{-7}$ - $4.4x10^{-4}$ M) caused a concentration-dependent relaxation in both ileal longitudinal segments and urinary bladder rings. Intravenous doses of resveratrol (0.028-8.5 mg/kg) caused a dose-dependent fall of systolic and diastolic blood pressure of anesthetized rats. Oral administration of this compound (1.3, 4.0. 13 and 40 mg/kg) decreased carrageenan-induced paw edema in rats. Furthermore, oral administration of resveratrol (3, 10, 30 and 100 mg/kg) decreased the number of writhes induced by i.p. injection of 0.8% acetic-acid in mice. These effects of resveratrol and its potential as a medicinal source were discussed in the light of the reported effects of resveratrol on nitric oxide and prostaglandin synthesis.

الملخص

استخدمت نبتة العليق (*Smilax aspera*)، التي تنتمي للعائلة الزنبقية، في طب الأعشاب لمعالجة العديد من الأمراض. وقد أدى التحليل الكيميائي لهذه النبتة إلى عزل مركب ترانس-رزفيراترول. في هذا البحث، درست تأثيرات رزفيراترول على اللفائفي والمثانة البولية وعلى ضغط الدم والالتهاب في الجرذ، وعلى الألم في الفأر. أحدث رزفيراترول بتراكيز تتراوح من 3×10^{-5} إلى 1×10^{-1} ملغم/مل (تعادل 1.3×10^{-7} إلى 4.4×10^{-4} جزيئي) انبساطاً معتمداً على التركيز في قطع اللفائفي الطولية وفي حلقات المثانة البولية المعزولتين من الجرذ. وأحدثت جرعات من رزفيراترول حقنت بالوريد وتتراوح بين 0.028 إلى 8.5 ملغم/كغم انخفاضاً معتمداً على التركيز في الضغط الانقباضي والانبساطي في الجرذان المخدرة. كذلك، فإن جرعات عن طريق الفم للجرذان مقدارها 1.3، 4، 13، 40 ملغم /كغم من رزفيراترول قللت من حجم الاستسقاء الناتج عن حقن مادة كراجينين في كف الجرذ. فضلاً عن ذلك، فإن جرعات من رزفيراترول أعطيت عن طريق الفم للفأر مقدارها 3، 10، 30، 100 ملغم/كغم قللت من عدد مرات التلوي بسبب الألم الناتج عن حقن 0.8 % من حامض الخليك داخل الصفاق. تم مناقشة تأثيرات رزفيراترول هذه وآفاق استخدامه كمادة علاجية في ضوء المعلومات المدونة عنه في الأدب العلمي والمتعلقة بتحرر حامض النتريك وتحرر البروستاغلانديناتز.

Keywords: Resveratrol; *Smilax Aspera*; Smooth Muscle; Blood Pressure; Inflammation; Analgesia.

1. Introduction

Smilax aspera is an evergreen, creeping, extremely tough shrub that belongs to the Liliaceae family (Longo and Vasapollo, 2006) and has been used in herbal medicine for muscle relaxation, skin ailment, rheumatic pain, depurative, diuretic, diaphoretic, antigout, dropsy, stimulant and for its tonic properties (Longo and Vasapollo, 2006; Aburjai *et al*., 2007). *S. aspera* has also been used traditionally for the treatment of syphilis (Vermani and Garg, 2002), diabetes (Fukunaga *et al*., 1997), rheumatism (Ageel *et al*., 1989), as an antioxidant (Demo *et al*., 1998) and to treat symptoms of menopause in women (Weil *et al*., 2000). In fact, several species of the genus *Smilax* are well-known Chinese traditional medicines, and are used as anti-inflammatory, anticancer and analgesic agents (Shu *et al*., 2006). Chemical and pharmacological studies were carried out on this species (e.g. Ageel *et al.*, 1989; Ruan *et al*., 2005; Selenu *et al*., 2005; Xu *et al*., 2005; Longo and Vasapollo, 2006). In an attempt to isolate chemical ingredients from the local *Smilax aspera*, we have been able to isolate the known stilbene trans-resveratrol.

Resveratrol is a non flavonoid polyphenolic compound found in a large number of plant species (at least 72 species), a number of which are human diet components, including mulberries, peanuts, grapes, and red wines (Lastra and Villegas, 2005). Resveratrol has been proposed as an effective agent in the prevention of pathologic processes such as inflammation, atherosclerosis, oxidative stress and carcinogenesis (Miatello *et al*., 2005; Baur and Sinclair, 2006). It has also been reported as neuroprotective, cardioprotective and to modulate lipoprotein metabolism (Martin *et al*., 2004; Baur *et al*., 2006). The interest in resveratrol was rekindled after reports that it extends life span in the yeast *Saccharomyces cervisiae* (Howitz *et al*., 2003) and the worm *Caenorhabditis elegans* and the fruit fly *Drosophila*

* Corresponding author. Shtaywy@ju.edu.jo.

melanogaster (Wood *et al*., 2004). In spite of reports to the contrary (Bass *et al*., 2007; Pearson *et al*., 2008), the debate on the biological effects of resveratrol continued to generate scientific interest (Valenzano *et al*., 2006). This communication is a contribution to evaluate other potential effects of resveratrol such as its effects on isolated smooth muscle preparations, blood pressure, and inflammation in rats and on pain in mice.

2. Materials and Methods

2.1. Effect of Resveratrol on Isolated Ileum and Urinary Bladder

Male and female albino rats weighing 250-300gm were used. Animals were lightly anesthetized with ether, and then sacrificed in compliance with the university adopted guidelines for animals' use in research. A mid piece of the ileum was excised, flushed with aerated physiological salt solution (PSS) and cut into several pieces (1-2cm long each) (Hammad and Abdalla, 1997). Urinary bladder was also isolated, and cut into 2-3mm rings taken from the middle region of the bladder (Khattab and Al-Hrasen, 2006). The preparations were placed in 10-ml water-jacketed glass tissue baths containing aerated PSS at 37^{o}C and gassed with a mixture of 95% O_2- 5% CO_2 and mounted for isometric recording using force transducers (Grass FTO3C) connected to a physiograph (Graphtec Thermal Arraycorder, WR5000) under a tension of 1g (Abdalla *et al*., 1994).

After an equilibration period, cumulative concentration-response curves of resveratrol were established by addition of 3-fold increasing concentration of resveratrol. When the maximum response to resveratrol was achieved, a strong non-specific relaxant agent (10^{-3} M papaverine HCl) was added to the tissue bath. The response to resveratrol was expressed as percent of the maximum effect of papaverine (Onwukaeme *et al*., 1999).

2.2. Effect of Resveratrol on Blood Pressure

Male albino rats (250-350 gm body weight) were anesthetized with sodium thiopental (50mg/kg body weight; i.p.). The right common carotid artery was exposed and a catheter was introduced for the recording of blood pressure using P23AA Statham pressure transducer situated at the level of the heart and connected to a Gilson polygraph (Abdalla *et al*., 1994). The right femoral vein was also catheterized for intravenous injection of resveratrol. After a steady baseline of blood pressure was obtained (about 15 minutes), resveratrol was injected intravenously in doses of 0.028, 0.085, 0.28, 0.85, 2.8, and 8.5 mg/kg body weight. The changes in systolic and diastolic blood pressure were observed and expressed as percent of their respective control values obtained before resveratrol injection.

2.3. Anti-Inflammatory Effect of Resveratrol

Male albino rats, weighing 130-200 gm were used. Animals were fasted for 24 hours before the beginning of the experiment with free access to water (Amanlou *et al*., 2005). Animals were divided into 6 groups each of eight animals as follow:

1. Control group: Received 0.9% NaCl.
2. Four treated groups: Received 1.3, 4, 13, and 40 mg/kg of resveratrol.
3. Positive control group: Received 10 mg/kg of indomethacin, a reference anti-inflammatory drug.

All doses were administered orally at a dose volume of 0.5ml/100 g animal. One hour later, acute paw edema was induced by plantar injection of 0.1ml of 1% freshly prepared carrageenan suspension in normal saline into the right hind paw of each rat. The paw volume was measured before (0 h) and at intervals of 1, 2, 3, 4, 5, and 6 hrs after carrageenan using a plethysmometer (Type 7140 Ugo Basile, Italy) (Wu *et al*., 2006).

2.4. Antinociceptive Effect of Resveratrol

Male and female albino mice, weighing 20-30g were used. Animals were deprived of food, but with free access to drinking water for 12 hours prior to the experiments (Costa-Lotufo *et al*., 2004). The animals were divided into 6 groups each of eight animals as follows:

1. *Control group:* received 0.9% NaCl.
2. *Four treated groups:* received 3, 10, 30, and 100 mg/kg of resveratrol.
3. *Positive control group:* received 10 mg/kg of indomethacin, a reference analgesic drug.

Animals were pretreated orally with resveratrol 60 minutes prior to i.p. injection with 0.1ml/10g body weight of 0.8% (v/v) acetic acid solution in 0.9% NaCl (Rabelo *et al*., 2003). The number of writhes was counted during a 30-min period following the injection of acetic acid. A writhe was defined as a contraction of the abdominal muscles accompanied by an extension of the forelimbs and elongation of the body (Choi *et al*., 2005).

2.5. Solutions

The composition of PSS in mM was: NaCl 118.0; KCl 4.7; $CaCl_2.2H_2O$ 2.5; $MgCl_2$ 0.5; NaH_2PO_4 1.0; $NaHCO_3$ 24.0; and glucose 11.1. Trans-resveratrol was isolated from *Smilax aspera* and was identified by comparison of its spectral data with literature (Vitrac *et al*., 2005). Stock solution of resveratrol was prepared daily by dissolving resveratrol in minimal amount of 1N NaOH, the volume was then completed to 1ml with 0.9% NaCl (final concentration of NaOH was 0.075%). Papaverine HCl (Acros Organics, New Jersey) solution (10^{-1}M) was prepared by dissolving papaverine hydrochloride in distilled water. Indomethacin (Sigma-Aldrich Chemie, Germany) was prepared by dissolving in 0.5% carboxymethylcellulose sodium. Carrageenan (Fluka Biochemika, Switzerland) was prepared by dissolving 0.1 g of carrageenan in 10 ml 0.9%NaCl.

2.6. Statistical Analysis

Data are presented as means ± the standard error of means (SEM). One way-analysis of variance (ANOVA) and Student *t*-test for independent samples were used to screen for differences between the means of the samples. The differences were considered significant if $P < 0.05$. The median effective concentration producing 50% of the maximum response (EC50) was calculated from the plot. Experimental data were analyzed by a computer fitting treatment using GraphPad Prism 5.0 software.

3. Results

3.1. Effect of Resveratrol on Isolated Smooth Muscle Preparations

Figure 1 shows that $3x10^{-5}$ -$1x10^{-1}$ mg/ml resveratrol (=$1.3x10^{-7}$ - $4.4x10^{-4}$ M), caused concentration-dependent relaxation in the rat isolated ileal longitudinal segments. The EC50 of resveratrol for relaxation of ileal longitudinal segments was (8.0 ± 2.3) x 10^{-3} mg/ml (n=4).

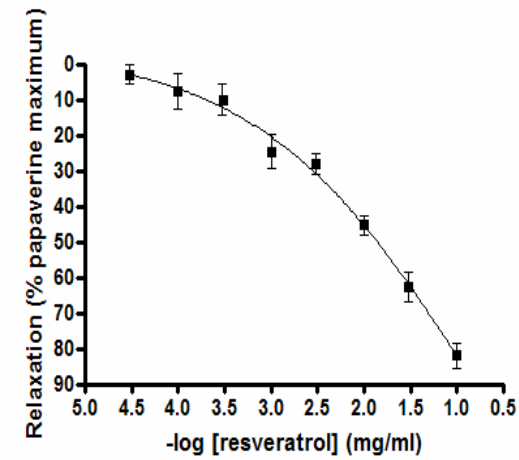

Figure 1. Cumulative concentration- response curves of resveratrol on rat isolated ileal longitudinal segments.

Figure 2 shows that resveratrol ($3x10^{-5}$ -$1x10^{-1}$ mg/ml) caused a concentration-dependent relaxation of the urinary bladder rings. The maximum relaxation induced by resveratrol reached (82.0 ± 4.1) % and (43.0 ± 6.5) % for ileal longitudinal segments and urinary bladder rings, respectively.

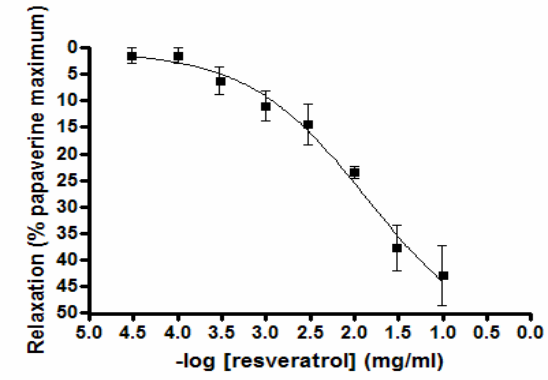

Figure 2. Cumulative concentration-response curves of resveratrol on rat isolated urinary bladder rings.

3.2. Hypotensive Effect of Resveratrol

Intravenous injection of resveratrol at doses ranging from 0.028 to 8.5 mg/kg induced dose-dependent fall of both systolic and diastolic blood pressure of the anesthetized rats (Figs. 3 and 4).

3.3. Anti-Inflammatory Effect of Resveratrol

Table 1 shows that resveratrol in doses of 1.3 and 4.0 mg/kg caused an inhibition in the hind paw volume over time although the decrease in the volume at these two

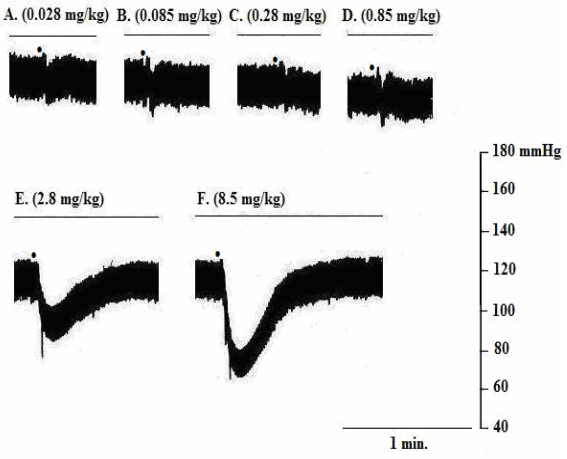

Figure 3. Typical responses of blood pressure to intravenous injections of resveratrol in the doses indicated (A-F). A-F were obtained from the same preparation. The solid line above the traces is a reference baseline.

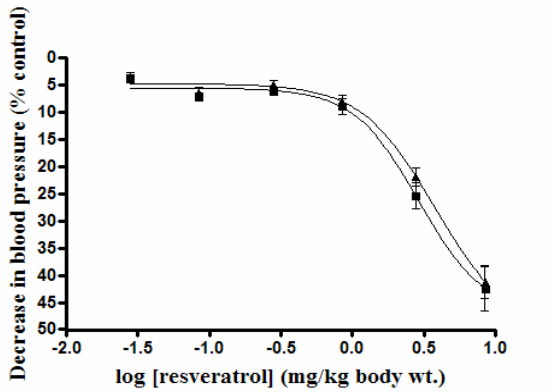

Figure 4. Dose-response curves of resveratrol on systolic (solid squares) and diastolic (solid triangles) blood pressure of anesthetized rats.

doses did not reach the level of statistical significance when compared to the control group. At concentrations of 13 and 40 mg/kg, there was a significant decrease in the hind paw volume, when compared to the control group. The positive control group also showed reduction of the volume of the hind paw and this inhibition reached the level of statistical significance 4, 5 and 6 hours after carrageenan administration.

3.4. Analgesic Effect of Resveratrol

Figure 5 shows that resveratrol-treated groups showed a dose-dependent decrease in the number of writhes counted in 30 minutes after injection of acetic acid. The highest reduction was obtained at a dose of 100 mg/kg (76.6 % of inhibition). This effect is comparable to that induced by indomethacin (71.3 % of inhibition) albeit a higher dose of resveratrol. Other resveratrol-treated groups (3, 10, and 30 mg/kg) showed intermediate inhibition values of 39.3 %, 54.0 %, and 56.6 %; respectively. This inhibition in the number of writhes reached the level of statistical significance at all doses of resveratrol-treated groups.

Table 1. Hind paw volumes in male rats observed over a period of six hours after oral administration of 0.9% NaCl, 1.3, 4, 13, and 40 mg/kg resveratrol, and indomethacin [a].

Experimental group	Zero time	1h	2h	3h	4h	5h	6h
Control (0.9% NaCl)	1.01±0.05	1.24±0.02	1.29±0.03	1.35±0.04	1.37±0.09	1.37±0.06	1.40±0.06
Resveratrol (1.3 mg/kg)	1.10±0.05	1.26±0.05	1.30±0.06	1.32±0.05	1.35±0.04	1.36±0.05	1.35±0.05
Resveratrol (4 mg/kg)	1.09±0.04	1.21±0.05	1.26±0.05	1.30±0.04	1.25±0.05	1.27±0.05	1.25±0.05
Resveratrol (13 mg/kg)	0.90±0.04	1.10±0.03	1.14±0.04	1.14±0.03*	1.12±0.02*	1.13±0.03*	1.06±0.03*
Resveratrol (40 mg/kg)	0.94± 0.03	1.03±0.03*	1.01±0.03*	1.03±0.03*	1.09±0.02*	1.07±0.02*	1.06±0.03*
Indomethacin (10 mg/kg)	1.06±0.04	1.12±0.04	1.13±0.02	1.18±0.02	1.15±0.03*	1.14±0.03*	1.13±0.01*

[a]All values represent means of the volume ± SEM for 8experiments.
*P < 0.05 compared to the control group.

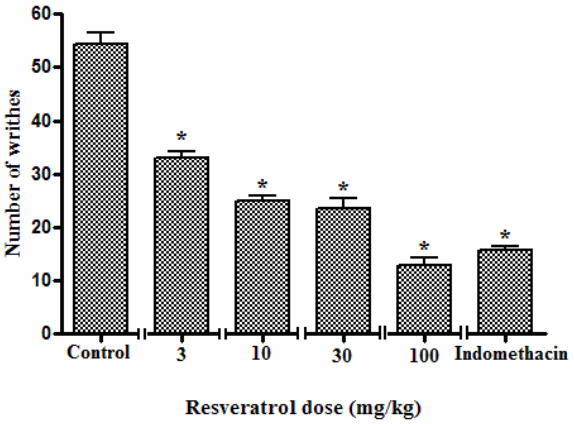

Figure 5: Number of writhes induced by acetic acid in mice treated with: 0.9% NaCl (control), 3, 10, 30, and 100 mg/kg resveratrol, and with 10 mg/kg indomethacin. Number of writhes is given as the means ± SEM of 8 animals. Asterisks indicate that P < 0.05 compared to the control group (0.9% NaCl).

4. Discussion

The growing interest in the use of herbs and their bioactive compounds is attributed largely to the fact that most herbs are relatively inexpensive and are easily available, yet have fewer adverse effects (Kim *et al*., 2006). *Smilax aspera,* a member of the Liliaceae family, is well known for its medicinal uses and has been used for many disorders. Many constituents have been isolated from this plant including flavonoids, tannins, asperoside, sapogenins, β-sitosterol and essential oils (Ayengar and Rangaswami, 1967; Petricic and Radosevic, 1969; Tschesche *et al*., 1974; Bruno *et al*., 1985; Long and Vasapollo, 2006). The current study on the whole plant material of *Smilax aspera* yielded resveratrol (0.0067% yield) which has been isolated from the genus *Smilax* previously (Zhang *et al*., 2006).

Resveratrol has been proposed as an effective agent in the prevention of pathologic processes such as inflammation, atherosclerosis, oxidative stress, and carcinogenesis (Miatello *et al*., 2005). Resveratrol, a polyphenolic compound found in many edible plant species, is satisfactorily absorbed from the intestine and distributed in the blood stream and can be detected in significant concentrations in the blood and a number of organs. Little is known about the transport and the distribution of resveratrol through the body but it must be bound to proteins and/or be conjugated to remain at high concentration in the serum as a consequence of its relatively low water solubility (Lastra and Villegas, 2005). Therefore, its pharmacological effects and its pharmacokinetics are of paramount importance to human health.

4.1. Resveratrol Spasmolytic Effects

In the current study, resveratrol has been found to cause concentration-dependent relaxation in both rat ileal longitudinal segments and urinary bladder rings. This observation is consistent with other studies which showed relaxant effects for resveratrol on isolated smooth muscles preparations. The relaxant effect of this compound was documented in many vascular beds including rat aorta, porcine coronary arteries, guinea pig mesenteric and uterine arteries and sheep coronary arteries (Naderali *et al*., 2000; El-Mowafy, 2002; Slater *et al.*, 2003; Campos-Toimil *et al.*, 2005; Buluc and Demirel-Yelmaz, 2006; King *et al*., 2006).

The mechanism of resveratrol relaxation cannot be deduced from the current experiments but observations made by other workers are suggestive of such mechanism. In one hand, several studies showed that nitric oxide relaxes ileum and urinary bladder via increasing cGMP synthesis (Hedlund, 2005; Gharib-Naseri *et al*., 2007) and that resveratrol relaxed endothelium-intact rat aortic rings constricted by phenylepherine and KCl via NO release (King *et al*., 2006). On the other hand, resveratrol stimulates the endothelium-dependent vasorelaxation through the stimulation of the NO/cGMP cascade in rat aortic rings (Fitzpatrick *et al*., 1993; Slater *et al.*, 2003), and it was shown that resveratrol upregulates the GC/cGMP system in coronary artery smooth muscle and to

trigger a vasorelaxant response (El-Mowafy, 2002). The GC/cGMP system plays a key role in spasmolytic signaling mechanisms (Song *et al*., 2006). NO, synthesized by the enzyme NO synthase, is released by peripheral nerves and mediates relaxant effect on vascular and non-vascular smooth muscles (Capasso *et al*., 2004). NO produces smooth muscle relaxation by activating the soluble guanylate cyclase (sGC) and thereby increasing tissue levels of cGMP, which in turn interacts with various intracellular components that regulate activities of the contractile proteins (Hedlund, 2005). Other effects of resveratrol pertains to variable effects on Ca in various tissues and cells are also possible and may play a role in the mechanism of action of resveratrol (Buluc and Demirel-Yilmaz, 2006; Wang and Scherer, 2008).

4.2. Resveratrol Effect on Blood Pressure

In this study, resveratrol was found to reduce arterial blood pressure of anesthetized normotensive rats when administered intravenously, indicating that it has a hypotensive effect. Although one component of the effect of resveratrol on both systolic and diastolic pressure was transient, blood pressure, in general, did not return to the original baseline. The present work is consistent with the recent studies that demonstrated the effects of resveratrol on blood pressure in various *hypertensive* rat models. It has been shown that resveratrol decreased systolic and diastolic blood pressure in ovariectomized rats (Li *et al*., 2006), and in the partially nephroctomized rats (Liu *et al*., 2005). It is likely that the hypotensive effect of resveratrol in the present study is due to an increase in NO production from the vascular endothelium. This suggestion stems from the observations that resveratrol administration prevented blood pressure elevation and reduced both vascular and myocardial endothelial NO synthase (eNOS) activity in fructose-fed rats (Miatello *et al*., 2005). Moreover, resveratrol was shown to restore the eNOS activity in the rat aorta and to normalize blood pressure of rats with modeled arterial hypertension (Gumanova *et al*., 2007). NO generated in endothelial cells by eNOS is known to play a major role in blood pressure control (Minuz, 2006).

4.3. Resveratrol Effect on Inflammation

Among the several models of acute inflammation, carrageenan-induced inflammation has been well established as a valid model to study free radicals generation in paw tissue after inflammatory states. The cellular and molecular mechanism of carrageenan-induced inflammation is well characterized, and this model of inflammation is a standard model to screen the anti-inflammatory activity of various experimental compounds (Bilici *et al*., 2002). Carrageenan-induced edema is caused by the release of a number of inflammatory mediators and is initiated by dilation of arterioles and an eventual increase in permeability of postcapillary venules resulting in exudation of inflammatory cells and fluids at the site of injury. Inhibition of this acute phase of inflammation will therefore ultimately abort the inflammatory process (Duwiejua *et al*., 2002). In this study, the edema induced in the rat hind paw by carrageenan was maximal at the 6th hour of its plantar injection. This time course of edema is consistent with that reported by Zakaria *et al*., (2006) but longer than that observed by Koo *et al*., (2006) who showed progressive paw volume reaching a maximum at 4 hrs, or that reported by Vasudevan *et al*., (2006) who showed maximum swelling after 3 hrs. Our results showed that resveratrol caused a dose-dependent decrease in the rat hind paw volume and this inhibition was statistically significant after 3h and 1h for doses of resveratrol of 13 and 40 mg/kg respectively. This observation is consistent with the finding that resveratrol isolated from *Smilax china* in a dose of 0.2 mg/kg/day inhibited the cotton pellet-induced granuloma in rats (Ruan *et al*., 2005). Resveratrol has also a significant suppressive effect on hind paw edema induced by egg albumin in bilateral adrenalectomized rats. The anti-inflammatory effects of resveratrol was found independent of adrenal gland but might be related to inhibition of inflammatory mediators and free radicals (Liu *et al*., 2006). Contrary to our observation, another study failed to demonstrate any anti-inflammatory effect of resveratrol using the carrageenan-induced paw edema model in which the administration of resveratrol at doses of 0.4, 2, 10, and 50 mg/kg did not reverse the swelling and edema, although they reversed the hyperalgesia induced by local tissue injury provoked by carrageenan (Gentilli *et al*., 2001). This contradiction might be attributed to differences in the route of resveratrol administration (oral vs. i.p.), the time between injection of resveratrol and injection of carrageenan (1hr vs. 30 min), differences in the instrument used for measurements (plethysmometer vs. analgesiometer) and the parameter measured (paw volume vs. paw circumference).

Several possibilities could account for the anti-inflammatory effects of resveratrol: i) inhibition of cyclooxygenase activity, since resveratrol has been shown to inhibit the induced production of PGE_2 in a dose-dependent manner in human peripheral blood leukocytes (Richard *et al*., 2005), and it decreased significantly the elevated levels of rat PGD_2 *in vivo*. In the *in vitro* and *in vivo* models, resveratrol decreased the expression of cyclooxygenase-2 (COX-2) (King *et al.,* 2006). Because COX-2 is the key enzyme catalyzing the rate-limiting step in PG biosynthesis, it is likely that at least part of the anti-inflammatory activity of resveratrol can be attributed to inhibition of COX-2 expression, ii) inhibition of the NO-generating pathway, since resveratrol has been shown to inhibit both the generation of NO and the expression of inducible nitric oxide synthase (iNOS) in activated macrophages and in cultured cells (Tsai *et al*., 1999). The expression of inducible isoform of NO synthase has been proposed as an important mediator of inflammation (Bilici *et al*., 2002). Resveratrol is known to inhibit the synthesis and release of pro-inflammatory mediators, inducible nitric oxide synthase (iNOS) and COX-2 via its inhibitory effects on nuclear factor – (kappa)B (NF- κB) (Sharma *et al*., 2007a). Tsai *et al*. (1999) showed that resveratrol protected against endotoxin-induced inflammation by preventing the NF- κB activation. In addition, resveratrol has been shown to be a potent and specific inhibitor of tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) and consequently the induced NF-κB activation (Fulgenzi *et al*., 2001).

4.4. Resveratrol Analgesic Effects

In this study, resveratrol was also shown to decrease the number of writhes in a dose-dependent manner. We used the acetic acid-induced mice abdominal writhing model to study the antinociceptive effect. Contortions induced by intraperitoneal injections of acetic acid originate from the pain of inflammation mediated by prostaglandins (Oliveira *et al*., 2001). When the tissues and cells suffer harmful stimulation, they release compounds such as H^+, prostaglandin E_2 (PGE_2) or 5-hydroxytryptamine (5-HT) which cause pain at the location. Acetic acid H^+ itself may cause pain; at the same time it can stimulate the tissue to produce PGE_2 which causes further pain. Thus, acetic acid is widely used to screen compounds for antinociceptive activity and is accepted as a suitable model for antinociception (Shu *et al*., 2006). Levels of prostaglandins were reported to be high during the first 30 min after intraperitoneal injection of acetic acid (Neto *et al*. 2005). Our observations are consistent with those by Torres-Lopez *et al.* (2002) which showed that resveratrol has peripheral antinociceptive effect since it reduced nociception in a dose-dependent manner in the second phase of formalin test which is dependent on many mediators including prostaglandins (Sayyah et al., 2002). In addition, resveratrol has been shown to prevent streptozotocin-induced thermal hyperalgesia in diabetic mice (Sharma *et al*., 2007b). Although we have not investigated the mechanism of action of resveratrol as antinociceptive in the present work, we postulate that resveratrol causes this effect through inhibition of prostaglandin synthesis through an action on COX enzyme since there is evidence that resveratrol produces antinociception through the selective inhibition of COX-1 and COX-2 (Grandos-Soto *et al*., 2002). Other workers showed that resveratrol has the ability to disrupt arachidonic acid metabolism by inhibiting COX-1 and hyperperoxidase activity of COX-1. Furthermore, resveratrol has been reported to inhibit COX-2 expression induced by carcinogens in human breast and oral epithelial cells (Tang *et al*., 2006) and to inhibit potently PGE_2 production and free radical scavenging in activated microglial cells (Candelario-Jalil *et al*., 2007). Other mechanisms of analgesia include the reduction of prostaglandin production at the primary afferent neurons by resveratrol thus diminishing the depolarization of nerve terminals otherwise observed during the nociception process. Alternatively, the opening of Ca^{2+}-activated K^+ channels by resveratrol would further reduce the depolarization leading to antinociception (Grandos-Soto *et al*., 2002). Also, resveratrol in doses of 5, 10, 20, and 40 mg/kg i.p. caused dose-dependent analgesia in the hot plate test and this effect was suggested to be mediated via an opioidergic mechanism (Gupta *et al*., 2004).

In conclusion, our results demonstrated that trans-resveratrol has spasmolytic, hypotensive, anti-inflammatory effects on rats and analgesic effects when tested in mice, and this compound could represent a source for the development of plant-based therapy useful in the control of arterial blood pressure, inflammation and pain. Further work is needed to elucidate the mechanism of action of this compound.

References

[1] Abdalla S, Abu Zarga M, Sabri S. 1994. Effects of flavone luteolin, isolated from *Colchicum ritchii*, on guinea pig smooth muscle and heart and on blood pressure and blood flow. Phytother. Res. 8(5): 265-270.

[2] Aburjai T, Hudaib M, Tayyem R, Yousef M, and Qishawi M. 2007. Ethnopharmacological survey of medicinal herbs in Jordan, the Ajloun Heights region. J. Ethnopharmacol. 110(2), 294-304.

[3] Ageel AM, Mossa JS, Al-Yahya MA, Al-Said MS, and Tariq M. 1989. Experimental studies on antirheumatic crude drugs used in Saudi traditional medicine. Drugs. Exp. and Clin. Res. 15(8): 369-372.

[4] Amanlou M, Dadkhah F, Salehnia A, and Farsam H. 2005. An anti-inflammatory and anti-nociceptive effects of hydroalcoholic extract of *Satureja khuzistanica* Jamzad extract. J. Pharm. Pharm. Sci. 8(1): 102-106.

[5] Ayengar KNN, and Rangaswami S. 1967. Asperagenin, a rare type of steroidal sapogenin with 25-hydroxyl group. Curr. Sci. 36(24): 653-655.

[6] Bass TM, Weinkove D, Houthoofd K, Gems D, Partridge L. 2007. Effects of resveratrol on life span in *Drosophila melanogaster* and *Caenorhabditis elegans*. Mech. Ageing. Dev. 128(10): 546-552.

[7] Baur JA, Pearson KJ, Price NL, Jamieson HA, Lerin C, Kalra A, Prabhu VV, Allard JS, Lopez-Lluch G, Lewis K, Pistell PJ, Poosala S, Becker KG, Boss O, Gwinn D, Wang M, Ramaswamy S, Fishbein KW, Spencer RG, Lakatta EG, Couteur DL, Shaw RJ, Navas P, Puigserver P, Ingram DK, Cabo RD, and Sinclair DA. 2006. Resveratrol improves health and of mice on a high-calorie diet. Nature. 444(7117): 337-342.

[8] Baur JA, and Sinclair DA. 2006. Therapeutic potential of resveratrol: in vivo evidence. Nat. Rev. Drug. Discov. 5(6): 493-506.

[9] Bilici D, Akpinar E, and Kiziltunc A. 2002. Protective effect of melatonin in carrageenan-induced acute local inflammation. Pharmacol. Res. 46(2): 133-139.

[10] Bruno S, De Laurentis N, Amico A, and Stefanizzi L. 1985. Flourescence spectra of some steroidal sapogenin fluorophors. Fitoterapia. 56(1): 39-41.

[11] Buluc M, and Demirel-Yilmaz E. 2006. Resveratrol decreases calcium sensitivity of vascular smooth muscle and enhances cytosolic calcium increase in endothelium. Vasc. Pharmacol. 44(4): 231-237.

[12] Campos-Toimil M, Elies J, and Orallo F. 2005. Trans- and cis- resveratrol increase cytoplasmic calcium levels in A7r5 vascular smooth muscle cells. Mol. Nutr. Food Res. 49(5): 396-404.

[13] Candelario-Jalil E, De Oliveira ACP, Gräf S, Bhatia HS, Hüll M, Muñoz E, and Fiebich BL. 2007. Resveratrol potently reduces prostaglandin E_2 production and free radical formation in lipopolysaccharide-activated primary rat microglia. J. Neuroinflammation. 4:25.

[14] Capasso R, Izzo AA, Romussi G, Capasso F, Tommasi ND, Bisio A, and Mascolo N. 2004. A secoisopimarane diterpenoid from *Salvia cinnabarina* inhibits rat urinary bladder contractility *in vitro*. Planta Med. 70(2): 185-188.

[15] Choi J, Lee KT, Choi MY, Nam JH, Jung HJ, Park SK, and Park HJ. 2005. Antinociceptive anti-inflammatory effect of

monotropein isolated from the root of *Morinda officinalis*. Biol. Pharm. Bull. 28(10): 1915-1918.

[16] Costa-Lotufo LV, De Lucena DF, Andrade-Neto M, Bezerra JNS, Leal LKAM, De Sousa FCF, and Viana GS. 2004. Analgesic, antiinflammatory and central depressor effects of the hydroalcoholic extract and fractions from *Aeolanthus suaveolens*. Biol. Pharm. Bull. 27(6): 821-824.

[17] Demo A, Petrakis C, Kefalas P, and Boskou D. 1998. Nutrient antioxidants in some herbs and mediterranean plant leaves. Food Res. Intl., 31(5), 351-354.

[18] Duwiejua M, Woode E, and Obiri D. 2002. Pseudo-akuammigine, an alkaloid from *Picralima nitida* seeds, has anti-inflammatory and analgesic actions in rats. J. Ethnopharmacol. 81(1): 73-79.

[19] El-Mowafy AM. 2002. Resveratrol activates membrane-bound guanylyl cyclase in coronary arterial smooth muscle: a novel signaling mechanism in support of coronary protection. Biochem. Biophys. Res. Comm. 291(5): 1218-1224.

[20] Fitzpatrick DF, Hirschfield SL, and Coffey RG. 1993. Endothelium-dependent vasorelaxing activity of wine and other grape products. Am. J. Physiol. 265(2): H774-H778.

[21] Fukunaga T, Miura T, Furuta K, and Kato A. 1997. Hypoglycemic effect of the rhizome of *Smilax glabra* in normal and diabetic mice. Biol. Pharm. Bull. 20(1): 44-46.

[22] Fulgenzi A, Bertelli AAE, Magni E, Ferrero E, and Ferrero ME. 2001. *In vivo* inhibition of TNF α-induced vascular permeability by resveratrol. Transplant. Proc. 33, 2341-2343.

[23] Gentilli M, Mazoit JX, Bouaziz H, Fletcher D, Casper RF, Benhamou D, and Savouret JF. 2001. Resveratrol decreases hyperalgesia induced by carrageenan in the rat hind paw. Life Sci. 68(11): 1317-1321.

[24] Gharib-Naseri MK, Asadi-Moghaddam M, and Bahadoram S. 2007. Antispasmodic effect of *Tecoma stans* (L.) Juss leaf extract on rat ileum. DARU. 15(3): 123-128.

[25] Granados-Soto V, Argüelles CF, and Ortiz MI. 2002. The peripheral antinociceotive effect of resveratrol is associated with activation of potassium channels. Neuropharmacol. 43(5): 917-923.

[26] Gumanova N, Artyushkova E, Metel'skaya V, Kochkarov V, Pokrovskaya T, Danilenko L, Korneev M, Pokrovskii M, and Pashin E. 2007. Effect of antioxidants pQ510 and resveratrol on regulatory function of the endothelium in rats with modeled arterial hypertension. Bull. Exp. Biol. Med. 143(6): 619-622.

[27] Gupta YK, Sharma M, and Briyal S. 2004. Antinociceptive effect of trans-resveratrol in rats: involvement of an opioidergic mechanism. Methods Find. Exp. Clin. Pharmacol. 26(9): 667.

[28] Hammad HM, and Abdalla SS. 1997. Pharmacological effects of selected flavonoids on isolated ileum: structure-activity relationship. Gen. Pharmacol. 28(5): 767-771.

[29] Hedlund P. 2005. Nitric oxide/ cGMP-mediated effects in the outflow region of the lower urinary tract: is there a basis for pharmacological targeting of cGMP. World J. Urol. 23(6): 362-367.

[30] Howitz KT, Bitterman KJ, Cohen HY,et al. 2003. Small molecule activators of sirtuins extend *Saccharomyces cervisiae* lifespan. Nature. 425(6954): 191-196.

[31] Khattab MM, Al-Hrasen MN. 2006. Contractile activity ATP and diadenosine tetraphosphate on urinary bladder in the rats: role of superoxide anion and urothelium. Auton. Autacoid Pharmacol. 26(2): 149-156.

[32] Kim MJ, Lee HJ, Wiryowidagdo S, and Kim HK. 2006. Antihypertensive effects of *Gynura procumbens* extract in spontaneously hypertensive rats. J. Med. Food 9(4): 587-590.

[33] King RE, Bomser JA, and Min DB. 2006. Bioactivity of resveratrol. CRFSFS, 5(3): 65-70.

[34] Lastra CA, and Villegas I. 2005. Resveratrol as an anti-inflammatory and anti-aging agent: Mechanisms and clinical implications. Mol. Nutr. Food Res. 49(5): 405-430.

[35] Li H, Yao X, Zheng T, Li W, and Jia Z. 2006. Effects of resveratrol on arterial blood pressure and pressor response in ovariectomized rats. J. Lanzhou Univ. 42(6), 50-54.

[36] Liu F, Qu JB, Li H, and Yang SJ. 2006. The anti-inflammatory mechanisms of resveratrol. Zhongguo Zhongyao Zazhi. 41(15): 1138-1141.

[37] Liu Z, Song Y, Zhang X, Liu Z, Zhang W, Mao W, Wang W, Cui W, Zhang X, Jia X, Li N, Han C, and Liu C. 2005. Effects of *trans*-resveratrol on hypertension-induced cardiac hypertrophy using the partially nephrectomized rat model. Clin. Exp. Pharmacol. Physiol. 32(12): 1049-1054.

[38] Longo L, and Vasapollo G. 2006. Extraction and identification of anthocyanins from *Smilax aspera* L. berries. Food Chem. 94(2): 226-231.

[39] Martin AR, Villegas I, La Casa C, Alarcon DL, and Lastra C. 2004. Resveratrol, a polyphenol found in grapes, suppresses oxidative damage and stimulates apoptosis during early colonic inflammation in rats. Biochem. Pharmacol. 67(7): 1399-1410.

[40] Miatello R, Vázquez M, Renna N, Cruzado M, Zumino A, and Risler N. 2005. Chronic administration of resveratrol prevents biochemical cardiovascular changes in fructose-fed rats. Am. J. Hypertension. 18(6): 864-870.

[41] Minuz P. 2006. Antagonistic modulation of vascular tone by Rho-kinase and nitric oxide: new insights into the mechanisms of blood pressure control. J. Hypertension. 24(7): 1253-1255.

[42] Naderali EK, Doyle PJ, and Williams G. 2000. Resveratrol induces vasorelaxation of mesenteric and uterine arteries from female guinea-pigs. Clin. Sci. 98: 537-543.

[43] Neto A, Costa J, Belati C, Vinhólis A, Possebom L, Filho ADS, Cunha W, Carvalho J, Bastos J, and Silva M. (2005). Analgesic and anti-inflammatory activity of a crude root extract of *Pfaffia glomerata* (Spreng) Pedersen. J. Ethnopharmacol. 96(1-2): 87-91.

[44] Oliveira AC, Leal-Cardoso JH, Santos CF, Morais SM, and Coelho-de-Souza AN. 2001. Antinociceptive effects of the essential oil of *Croton zehntneri* in mice. Braz. J. Med. Biol. Res. 34(11): 1471-1474.

[45] Onwukaeme ND, Lot TY, and Udoh FV. 1999. Effects of *Daniellia oliveri* bark on isolated rat bladder. Phytother. Res. 13(5): 416-418.

[46] Pearson KJ, Baur, JA, Lewis, K et al. 2008. Resveratrol delays age- related deterioration and mimics transcriptional aspects of dietary restriction without extending lifespan. Cell Metab. 8(2): 157-168.

[47] Petricic J, and Radosevic A. 1969. Asperoside, a new bisdesmosine 22-hydroxyfurostanol saponin from *Smilax aspera*. Farmaceutski Glasnik 25(3): 91-95.

[48] Rabelo M, Souza EP, Soares PMG, Miranda AV, Matos FJA, Criddle DN. 2003. Antinociceptive properties of the essential

oil of *Ocimum gratissimum* L. (Labiatae) in mice. Braz. J. Med. Biol. Res. 36(4): 521-524.

[49] Richard N, Porath D, Radspieler A, and Schwager J. 2005. Effects of resveratrol, piceatannol, tri-acetoxystilbene, and genistein on the inflammatory response of human peripheral blood leukocytes. Mol. Nutr. Food Res. 49(5): 431-442.

[50] Ruan J, Zou J, and Cai Y. 2005. Anti-inflammatory components of *Smilax china.* Yivao Daobao. 24(8): 670-672.

[51] Sayyah M, Peirovi A, and Kamalinejad M. 2002. Anti-nociceptive effect of the fruit essential oil of *Cuminum cyminum L.* in rat. Iran. Biomed. J. 6(4): 141-145.

[52] Selenu MB, Carrus F, and Bonsignore L. 2005. Ethnopharmacobotany of east-central Sardinian: chemical and biological study on *Smilax aspera*. Boll. Chim. Farm. 144(1): 79-92.

[53] Sharma S, Kulkarni S, and Chopra K. 2007a. Effect of resveratrol, a polyphenolic phytoalexin, on thermal hyperalgesia in a mouse model of diabetic neuropathic pain. Fundam. Clin. Pharmacol. 21(1): 89-94.

[54] Sharma S, Chopra K, and Kulkarni SK. 2007b. Effect of insulin and its combination with resveratrol or curcumin in attenuation of diabetic neuropathic pain: participation of nitric oxide and TNF-alpha. Phytother. Res. 21(3): 278-283.

[55] Shu XS, Gao ZH, and Yang XL. 2006. Anti-inflammatory and anti-nociceptive activities of *Smilax china* L. aqueous extract. J. Ethnopharmacol. 103(3): 327-332.

[56] Slater SJ, Seiz JL, Cook AC, Stagliano BA, and Buzas CJ. 2003. Inhibition of protein kinase C by resveratrol. Biochim. Biophys. Acta.1637(1): 59-69.

[57] Song LH, Pan W, Yu YH, Quarles LD, Zhou HH, and Xiao ZS. 2006. Resveratrol prevents CsA inhibition of proliferation and osteoblastic differentiation of mouse bone marrow-derived mesenchymal stem cells through an ER/NO/cGMP pathway. Toxicol. In Vitro 20: 915–922.

[58] Tang HY, Shih A, Cao HJ, Davis FB, Davis PJ, and Lin HY. 2006. Research articles: therapeutics: resveratrol-induced cyclooxygenase-2 facilitates p53-dependent apoptosis in human breast cancer cells. Mol. Cancer Ther. 5(8): 2034-2042.

[59] Torres-López JE, Ortiz MI, Castañeda-Hernădez G, Alonso-López R, Asomoza-Espinosa R, and Granados-Soto V. (2002). Comparison of the antinociceptive effect of celecoxib, diclofenac and resveratrol in the formalin test. Life Sci. 70(14): 1669-1676.

[60] Tsai SH, Lin-Shiau SY, and Lin JK. 1999. Suppression of nitric oxide synthase and down-regulation of the activation of NFκB in macrophages by resveratrol. Br. J. Pharmacol. 126(3): 673-680.

[61] Tschesche R, Harz A, and Petricic J. 1974. Steroid saponins with more than one sugar chain. VIII. asperoside, a bisdesmosidic 22-hydroxyfurostanol glycoside of *Smilax aspera*. Chem. Ber. 107(1): 53-61.

[62] Valenzeno DR, Terzibasi E, Genade T, Cattaneo A, Domenici L, Cellerino A. 2006. Resveratrol prolongs lifespan and retards the onset of age-related markers in a short-lived vertebrate. Curr. Biol. 16(3): 296-300.

[63] Vasudevan M, Gunnam KK, and Parle M. 2006. Antinociceptive and anti-inflammatory properties of *Daucus carota* seeds extract. J. Health Sci. 52(5), 598-606.

[64] Vermani K, and Garg S. 2002. Herbal medicines for sexually transmitted diseases and AIDs. J. Ethnopharmacol. 80(1): 49-66.

[65] Vitrac X, Bornet A, Vanderlinde R, Valls J, Richard T, Delaunay JC, Merillon JM, and Teissedre PL. 2005. Determination of stilbenes (δ-viniferin, trans-astringin, trans-piceid, cis- and trans-resveratrol, ε-viniferin) in Brazilian wines. J. Agric. Food Chem. 53(14): 5664-5669.

[66] Wang ZV, and Scherer PE. 2008. Adiponectin, cardiovascular function, and hypertension. Hypertension, 51(12): 8-14.

[67] Weil V, Cirigliano M, and Battistini M. 2000. Herbal treatments for symptoms of menopause. Clin. Pers. Complem. Med. 35-44.

[68] Wood JG, Rogina B, Lavul S, Helfand SL, Tatar M, Sinclair D. 2004. Sirtuin activators mimic calorie restriction and delay aging in metazoans. Nature, 430(7000): 686-689.

[69] Wu Y, Zhou C, Li X, Song L, Wu X, Lin W, Chen H, Bai H, Zhao J, Zhang R, Sun H, and Zhao Y. 2006. Evaluation of antiinflammatory activity of the total flavonoids of *Laggera pterodonta* on acute and chronic inflammation models. Phytother. Res. 20(7): 585-590.

[70] Xu J, Xian L, Zhang P, Li ZL, and Wang Y.2005. Antiinflammatory constituents from the roots of *Smilax bockii* warb. Arch. Pharm. Res. 28(4): 395-399.

[71] Zakaria ZA, Sulaiman MR, Jais AMM, Somchit MN, Jayaraman KV, Balakhrisnan G, and Abdulla FC. 2006). The antinociceptive activity of *Muntingia calabura* aqueous extract and the involvement of L-arginine/nitric oxide/cyclic guanosine monophosphate pathway in its observed activity in mice. Fund. Clin. Pharmacol. 20(4): 365-372.

[72] Zhang CL, Wang DM, Zhu W, Zhang HI. 2006. Study on chemical constituents from stem of *Smilax scobinicaulis*. Zhongguo Zhongyao Zazhi. 41(20): 1540-1542.

JJBS
Jordan Journal of Biological Sciences

Volume 2, Number 2, June 2009
ISSN 1995-6673
Pages 77 - 82

Genetic Polymorphism of Manganese Superoxide Dismutase (MnSOD) Among Breast Cancer and Benign Breast Patients in Jordan: A Preliminary Study

Malak S. Abdel-Fattah[a,*], Manar Atoum[b], Saleem Abdel-Rahman[c], Ali Alobous[d]

[a]*Department of Biology and Biotechnology, Faculty of Sciences, Hashemite University, Zarqa, Jordan*

[b]*Department of Medical Laboratory Sciences, Faculty of Allied Health Sciences, Hashemite University, Zarqa, Jordan*

[c]*Department of Biology and Biotechnology, Faculty of Sciences, Hashemite University, Zarqa, Jordan*

[d]*MD senior consultant general and oncology, Royal Medical services(King Hussain Medical City),Amman, Jordan*

Abstract

This study investigated the association of MnSOD gene polymorphism with risk of both breast cancer and benign breast disorders among Jordanian females. MnSOD genotyping assay was conducted among 81 Jordanian participants including: 44 breast cancer patients, 19 benign breast patients and 18 healthy controls. In the breast cancer group, MnSOD allele frequency for Ala and Val alleles was 41% and 59%, respectively, which is similar to the Caucasian population. The most common MnSOD genotype among all study groups is Ala/Val genotype, while the Ala/Ala genotype was rare in benign breast patients and controls and was completely absent in breast cancer patients. Women with Ala/Val genotype were at highly significant increased risk for breast cancer (OR=10, 95% CI=2.51-39.83), while Val/Val genotype was correlated to a significantly decreased risk for breast cancer (OR=0.125, 95% CI=0.03-0.5). On the other hand, the similar risks for benign breast diseases were nonsignificant. Larger sample is needed to support that MnSOD polymorphism may be involved in breast cancer development and progression.

الملخص

لقد تم تقيم التعداد الشكلي لجين (MnSOD) لدى إناث أردنيات مصابات بسرطان الثدي الخبيث و اخريات مصابات بسرطان الثدي الحميد, بالاضافه الى دراسة إرتباط التعداد الشكلي لجين (MnSOD) مع قابلية الاصابه بسرطان الثدي الخبيث والحميد على حد سواء. تم تحديد الطراز الجيني للأنزيم (MnSOD) ل 81 عينة ضمت 44 مريضه بسرطان الثدي الخبيث, 19 مريضه بسرطان الثدي الحميد و 18 من الإناث الاصحاء. كانت نسبة تكرار الأليلين الخاصين بالأنزيم (MnSOD) ألانين و فالين في عينات الدراسه مجتمعة هي 41% و 59% على التوالي, والتي هي اكثر قربا من الشعب القوقازي. كان الطراز الجيني ألانين/فالين هو الاكثر شيوعا بين مجموعات الدراسه, بينما كان الطراز الجيني ألانين/ألانين نادر الانتشار لدى الإناث الأصحاء و الإناث المصابات بسرطان الثدي الحميد وغائبا تماما لدي المصابات بسرطان الثدي الخبيث. لقد وجد ان السيدات اللاتي يحملن الطراز الجيني ألانين/فالين هن الأكثر عرضة للاصابه بسرطان الثدي الخبيث بينما السيدات اللاتي يحملن الطراز الجيني فالين/فالين هن الأقل عرضة للإصابة بسرطان الثدي الخبيث. أما قابلية الاصابه بسرطان الثدي الحميد المرتبطة بالطراز الجيني لأنزيم (MnSOD) كانت نوعا ما قريبة من احتمالية الاصابة بسرطان الثدي الخبيث إلا انها لم تكن ذات ارتباط معنوي مثلها. استخلصت هذه الدراسه الى تأييد احتمالية ضلوع التعداد الشكلي لجين (MnSOD) بتطور سرطان الثدي الخبيث.

Keywords: Breast Cancer; Benign Breast Tumor; Manganese Superoxide Dismutase (MnSOD); Polymorphism; Reactive Oxygen Species (ROS).

1. Introduction

Breast cancer is the second most common type of cancer after lung cancer and the fifth most common cause of cancer death among both sexes worldwide (World Health Organization, 2006). Among women, breast cancer is the leader incident cancer and the second cause of death (Jemal *et al.,* 2005). In Jordan, breast cancer amounts to 32 % of the total number of cancer patients, with an average of 500 females and 5 males diagnosed with breast cancer every year, which is relatively high when compared to the overall size of the population (Al-Khatib, 2007).

Although benign breast conditions are not life-threatening as breast cancer, benign lesions of the breast are far more frequent than cancerous ones and can cause serious physical symptoms, a financial burden for health services and emotional problems for patients and families since certain benign conditions are linked with an increased risk of developing breast cancer (Guray and Sahin, 2006).

The etiology of breast cancer is multifactorial, both hormonal genetic and environmental factors are implicated in the pathogenesis of breast cancer (Russo *et al*., 2000). One potential mechanistic basis for these factors is through reactive oxygen species (ROS)-induced oxidative damage (Wang *et al.,* 2001; Mitrunen *et al.,* 2001; Millikan *et al.,* 2004; Cai *et al.,* 2004; Mukhopadhyay *et al.,* 2004 ; Waris and Ahsan, 2006).

* Corresponding author. malaksofyan@yahoo.com.

The mitochondrial respiratory chain constitutes the main intracellular source of ROS in most tissues (Julio, 2003), that makes the mitochondrial antioxidant defense "manganese-containing superoxide dismutase" liable to maintain the steady-state concentration of ROS at non-toxic levels. Therefore, it would be advantageous to pinpoint the biological role of manganese superoxide dismutase (MnSOD) and its signification in cancer development since there were many studies considered it as a tumor suppressor gene (Mukhopadhyay *et al.,* 2004).

MnSOD is encoded by a single gene, containing five exons and located on chromosomal region 6q25.3. Recently, a genetic variant of MnSOD was identified, a Thymine (T) to Cytosine (C) substitution in the mitochondrial targeting sequence, which changes the code of the amino acid Valine from (GTT) to that of Alanine (GCT), leading to structural alteration in the enzyme conformation (Shimoda-Matsubayashi *et al.,* 1996). This alteration may affect the cellular allocation of MnSOD into mitochondria; therefore the enzyme could leave mitochondria without full defense against superoxide radicals (Rosenblum *et al.,* 1996). Sutton *et al.* (2003) supported that Alanine Ala allele of MnSOD allows more efficient transport into mitochondrial matrix while MnSOD of Valine Val allele will be partially arrested in the inner mitochondrial membrane.

To date, several studies have examined the correlation between MnSOD polymorphism and breast cancer risk with inconsistent results. Ambrosone *et al*. (1999), and Mitrunen *et al.* (2001) and Cai *et al.* (2004) reported that having an Ala allele increased the risk of breast cancer. In contrast, Egan *et al*. (2003), Knight *et al.* (2004) and Gaudet *et al*. (2005) did not support such association. Millikan *et al.* (2004) and Tamimi *et al.* (2004) also did not support an overall association but they suggested that MnSOD polymorphism may modify breast cancer risk. But there were no reports evaluated the association between MnSOD polymorphism and benign breast disorders risks. So, the aim of this study is to screen MnSOD gene polymorphism among Jordanian breast cancer patients, benign breast patients and healthy women and investigate the association between MnSOD polymorphism and risk of both breast cancer and benign breast disorders.

2. Materials and Methods

2.1. Study Samples

A total of 81 individuals were included in this study. Informed consent form was explained and provided to each individual participated in this study for interview and samples collection. All samples were collected from King Hussein Medical City (KHMC) in Amman, in the period between June 2007 and May 2008.

Control group included 18 blood samples drawn from healthy Jordanian females without any history of breast cancer or previous benign breast problems, which were collected in the routine laboratory of KHMC. The age of these females ranged from 24 to 62 years (mean=40.1 years).

Test group included 44 blood samples (cancer group) collected from Jordanian female patients histopathologically diagnosed with breast cancer, the age of these patients ranged from 25 years to 75 years (mean=52.3 years). Another 19 blood samples (benign group) were optioned from Jordanian females who were histopathologicaly diagnosed with benign breast diseases, the age of these females ranged from 17 to 70 years (mean=41.79 years).

2.2. MnSOD Genotyping

Genomic DNA was extracted from whole blood samples, using Wizard Genomic DNA Purification Kit (Promega, USA). DNA extraction was performed in accordance with the manufacturer's instructions.

MnSOD genotyping was conducted using polymerase chain reaction-restriction fragment length polymorphism PCR-RFLP as described in (Cai *et al.,* (2004) but with some modification. PCR amplification was carried out in a total volume of 25 µl containing: 2 µl of genomic DNA, 0.8 µM of each primer (5'-ACCAGCAGGCAGCTGGCGCCGG-3' and 5'-GCGTTGATGTGAGGTTCCAG-3') (Alpha DNA, Montreal), 8.5 µl of nuclease-free water and 12.5 µl of 2X PCR Master Mix (Fermentas, USA) containing: 0.05 units/µl *Taq* DNA polymerase supplied in reaction buffer, 4 mM of $MgCl_2$ and 0.4 mM of each dNTPs [dATP, dCTP, dGTP, dTTP]. PCR was performed in thermal cycler (BIO-RAD, USA). The thermal profile involved initial denaturation at 95 ˚C for 15 min, followed by 35 cycles of denaturation (94 ˚C for 30s), annealing (58˚C for 30s), and extension (72 ˚C for 30s), and completed with a final extension at 72˚C for 7 min.

RFLP technique was accomplished to detect MnSOD polymorphism using a restriction endonuclease enzyme *Ngo*MIV (Promega, USA) which recognizes a short specific DNA sequence to cleave double-stranded DNA at specific site within the recognition sequence. Restriction reaction was performed in a total volume of 20 µl containing 11.8 µl nuclease-free water, 2 µl 10X restriction enzyme buffer, 0.2 µl acetylated bovine serum albumin, 4 µl PCR product DNA and 2 µl (1 unit) of *Ngo*MIV restriction enzyme. After mixing, the reaction mixture was incubated at 37 ˚C for 4 hrs. Restricted products were electrophoresed on 4% agarose gel stained with ethidium bromide at 100 v for 45 min and visualized under UV. Fragment patterns specific for MnSOD genotypes were: Ala/Val (18 bp, 89 bp and 107 bp) bands, Val/Val (107 bp) band and Ala/Ala (18 bp and 89 bp) which were no table in Figure (1).

2.3. Statistical Analysis

Statistical analysis was carried out using the Statistical Package for Social Sciences (SPSS 10.05; SPSS Inc, Chicago, IL, USA, 1999). Chi-square test was used to evaluate case-control differences for MnSOD genotype distribution among different groups where statistical significance was set at $P < 0.05$. The association between MnSOD genotypes and the development of both malignant and benign breast disorders was examined by calculating the odds ratios (OR) and 95% confidence intervals (CI).

3. Results

As shown in Table (1), Ala/Ala genotype of MnSOD was completely absent only in breast cancer group, and there was a large difference between the frequency of Val/Val and Ala/Val genotypes (9.1% and 90.9%), respectively. While in control group, Ala/Ala genotype accounts for 5.6% of control samples, and the difference between Val/Val and Ala/Val genotypes frequency was relatively small (44.4% and 50%), respectively. The genotype distribution was statistically significant when the cancer and control groups were compared.

In benign group, the frequency of Ala/Val genotype (73.7%) was higher than in control group (50%). Approximately, the frequency of Val/Val genotype in benign group (21%) was half the frequency in control group (44.4%), and the frequency of Ala/Ala genotype was equal in both groups. Differences of genotype distributions between benign and control groups did not reach statistical significance (P=0.302) as shown in Table (1).

In addition, cancer group was compared to benign group, most of cancer patients (90.9%) and benign patients (73.7%) appeared to have the Ala/Val genotype, while the Val/Val was found in 9.1% of cancer patients and 21% of benign patients. Although the Ala/Ala genotype was found in 5.3% of benign patients and was absent in cancer group, the distribution of genotypes was not significantly different between cancer and benign groups (P=0.118).

MnSOD allele frequencies among each study group and among whole individuals participated in this study were calculated using the formula described by Campbell (1996). Val allele was the dominant allele among all groups, the prevalence of Val allele was 55% among cancer group, 58% among benign group, and 69% among control group. The frequency of Val allele in the whole study participants was 59% (Figure 2).

The risk of breast cancer associated with MnSOD genotypes was evaluated in this study. Females with Ala/Val genotype had a 10-fold significant increase risk for breast cancer development (OR=10, 95% CI=2.51-39.83), while a highly significant decreased risk for breast cancer was associated with Val/Val genotype (OR=0.125, 95% CI=0.03-0.5). The risk for breast cancer development associated with Ala/Ala genotype could not be determined because the Ala/Ala genotype was not found in any breast cancer females. But using Chi-square test, there was no significant difference (P=0.115) between cancer patients and healthy controls with Ala/Ala genotype (Table 2).

On the other hand, non significant 2.8-fold increase risk for benign breast diseases was associated with Ala/Val genotype (OR=2.8, 95% CI=0.71-11.1). Also, there was no significant risk of benign breast diseases associated with neither Val/Val genotype (OR=0.33, 95% CI=0.08-1.41) nor Ala/Ala genotype (OR=0.94, 95% CI=0.055-16.33) (Table 3).

4. Discussion

MnSOD polymorphic alleles are widely variable with ethnicity, the frequency for Ala allele is 12% among Japanese (Shimoda-Matsubayashi *et al.,* 1996) and 14% among Chinese (Cai *et al.,* 2004) , whereas it is more common (41-55%) in the Caucasian population (Ambrosone *et al.,* 1999). In this study, MnSOD genotyping assay was conducted on 81 Jordanian participants including: 44 breast cancer patients, 19 benign breast patients and 18 healthy controls, the frequency of Ala allele was 41% (Figure 2) which is comparable to that in Caucasian population.

MnSOD genotyping revealed that Ala/Val genotype had the highest frequency distribution for cancer group, benign group and control group (90.9%, 73.7% and 50%), respectively (Table 1). Frequency of MnSOD genotypes was found to be significantly different between breast cancer patient and controls, where the Ala/Ala genotype was completely absent among breast cancer patient that could be due to ethnic variation. However, more investigations and large sample will be needed to shed additional light on the role of Ala/Ala genotype in the etiology of breast cancer.

Due to the absence of Ala/Ala genotype in breast cancer group, this study could not estimate the association between Ala/Ala genotype and breast cancer risk. However, the most remarkable breast cancer risk was associated with Ala/Val genotype compared with Val/Val genotype. A 10-fold significantly increased risk of breast cancer was found among women having the Ala/Val genotype, while women with the Val/Val genotype have a significant decreased risk of developing breast cancer (Table 2). This study also evaluated the risk of benign breast diseases associated with MnSOD polymorphism, 2.8- fold increased risk of benign breast diseases was associated with Ala/Val genotype, a decreased risk was associated to Val/Val genotype and no association was observed with Ala/Ala genotype, although none of these risks was significant (Table 3).

Our results were in agreement with the findings of Ambrosone *et al.* (1999), Mitrunen *et al.* (2001) and Cai *et al.,* (2004), which all based on that the Ala-containing MnSOD is transported more efficiently through the mitochondrial membrane (Sutton *et al.,* 2003), were explained by the possible highly active Ala-containing MnSOD that could cause accumulation of H_2O_2 in mitochondria at least in the absence of H_2O_2- scavenging enzymes such as glutathione peroxidase and catalase, which could lead to oxidative damage and consequently cancer. Thus, it will be interesting to evaluate MnSOD genotype-activity relationship in order to explain such results.

Acknowledgments

The authors are grateful to the staff of breast clinic in KHMC who kindly facilitate the process of data and samples collection especially, Wafa`a Halaseh, Dr. Mohammad Al-Badour, Dr. Ra`ed Marji, and Dr. Saleh Alhurani. Also Sausan Abdel-Daim and Colonel Saleh Malkawi deserve all thanks for their generous technical support in KHMC laboratory. With pleasure, we thank Dr. Qiuyin Cai for generously providing useful comments during the genotyping of MnSOD, Dr. Huda Al-hourani for helping during statistical analysis and Foad Zgool for the technical support during the laboratory work.

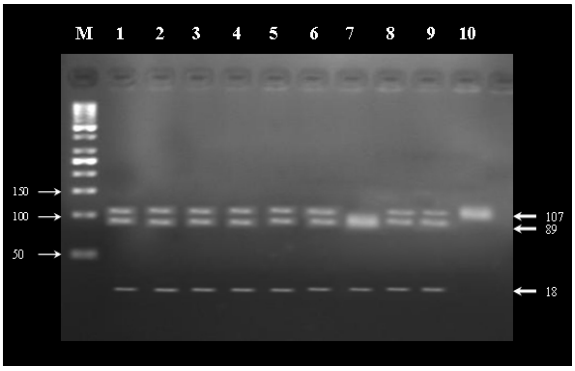

Figure 1. 4% agarose gel electrophoresis of selected genotyping assays. Lane M: 50 bp molecular weight ladder. Lanes 1,2,3,4,5,6,8 and 9 partially-digested samples showing three bands (107bp, 89bp and 18bp) representing Ala/Val genotype. Lane 7: a fully-digested sample showing two bands (89 bp and 18 bp) representing Ala/Ala genotype. Lane 10: undigested sample showing one 107 bp band representing Val/Val genotype.

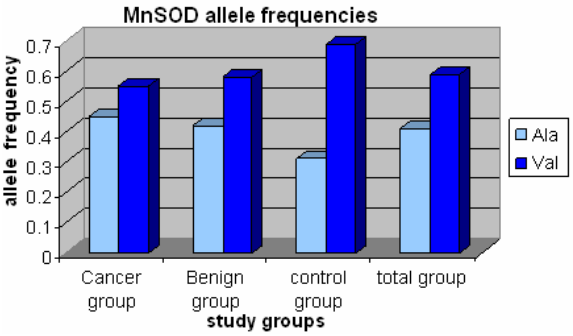

Figure 2. *MnSOD* allele frequencies among all study groups.

Table 1. Distribution of MnSOD genotypes among breast cancer patients, benign breast patients and control group.

MnSOD genotypes	Cancer group n = 44	Benign group n = 19	control group n =18
Ala/Ala	0	1 (5.3 %)	1 (5.6 %)
Val/Val	4 (9.1 %) *	4 (21.0 %)	8 (44.4 %)
Ala/Val	40 (90.9 %) *	14 (73.7 %)	9 (50.0 %)

Data presented as numbers of cases (percentage)
*: *P* value < 0.05 for cancer patients versus control differences

Table 2. Risk of breast cancer associated with MnSOD genotypes.

MnSOD genotypes	P value	odds ratios	95% confidence intervals
Ala/Ala	0.115		
Val/Val	0.001	0.125	0.03 - 0.50
Ala/Val	0.000	10	2.50 - 39.83

*: *P* value < 0.05 for gene polymorphism

Table 3. Risk of benign breast disease associated with MnSOD genotypes.

MnSOD genotypes	P *value*	*odds ratios*	*95% confidence intervals*
Ala/Ala	*0.969*	*0.94*	*0.055 - 16.33*
Val/Val	*0.129*	*0.33*	*0.08 - 1.41*
Ala/Val	*0.138*	*2.80*	*0.71 - 11.10*

*: *P* value < 0.05 for gene polymorphism

References

Al-Khatib, S. 2007. Breast cancer. 1st Levant Breast Cancer Summit. Novartis Oncology. Amman, Jordan.

Ambrosone, C., B. Freudenheim, J. L., Thompson, P. A., Bowman, E., Vena, J. E., Marshall, J. R., Graham, S., Laughlin, R., Nemoto, T. and Shields, P. G. 1999. Manganese superoxide dismutase (MnSOD) genetic polymorphisms, dietary antioxidants, and risk of breast cancer. *Cancer Res.,* 59: 602-606.

Cai, Q. Shu, X. O. Wen, W. Cheng, J. R. Dai, Q. Gao, Y. T. and Zheng, W. 2004. Genetic polymorphism in the manganese superoxide dismutase gene, antioxidant intake, and Breast cancer risk: results from Shanghai breast cancer study. *Breast Cancer Res.,* 6: R647-R655.

Campbell, N. A. 1996. Biology. Fourth edition. Benjamin/Cummings Publishing Co., California.

Egan, K. M. Thompson, P. A. Titus-Ernstoff, L. Moore, J. H. and Ambrosone, C. B. 2003. MnSOD polymorphism and breast cancer in a population-based case-control study. *Cancer Lett.,* 199(1):27-33.

Gaudet, M. M. Gammon, M. D. Santella, R. M. Britton, J. A. Teitelbaum, S. L. Eng, S. M. Terry, M. B. Bensen, J. T. Schroeder, J. Olshan, A. F. Neugut, A. I. and Ambrosone, C. B. 2005. MnSOD Val-9Ala genotype, pro- and anti-oxidant environmental modifiers, and breast cancer among women on Long Island, New York. *Cancer Causes Control,* 16(10):1225-34.

Guray, M. and Sahin, A. A. 2006. Benign Breast Diseases: Classification, Diagnosis, and Management. *The Oncologist*, 11(5):435-449.

Jemal, A. Murray, T. Ward, E. Samuels, A. Tiwari, R. C. Ghafoor, A. Feuer, E. J. and Thun, M. J. 2005. Cancer statistics. *CA Cancer J Clin,* 55 (1): 10–30.

Julio, T. F. 2003. Mitochondrial formation of reactive oxygen species. *J Physiol.*, 552(2): 335-344.

Knight, J. A. Onay, U. V. Wells, S. Li, H. Shi, E. J. Andrulis, I. L. and Ozcelik, H. 2004. Genetic variants of GPX1 and SOD2 and breast cancer risk at the Ontario site of the Breast Cancer Family Registry. *Cancer Epidemiol Biomarkers Prev.,* 13:146-149.

Millikan, R. C. Player, J. Cotret, A. R., Moorman, P. Pittman, G. Vannappagari, V. Tse, C. K. J. and Keku, T. 2004. Manganese superoxide dismutase Ala-9Val polymorphism and risk of breast cancer in a population-based case–control study of African Americans and whites. *Breast Cancer Res.earch*, 6 (4):R264-R274.

Mitrunen, K. Silanpaa, P. Kataja, V. Eskelinen, M. Kosma, V. Bennhamou, S. Uusitupa, M. and Hirvonen, A. 2001. Association between manganese superoxide dismutase (MnSOD) gene

polymorphism and breast cancer risk. *Carcinogenesis*, 22:827-829.

Mukhopadhyay, S. Das, S. K. and Mukherjee, S. 2004. Expression of Mn-superoxide dismutase gene in nontumorigenic and tumorigenic human mammary epithelial cells. *Journal of Biomedicine and Biotechnology*, 4 (2004):195–202.

Rosenblum, J. Gilula, N. and Lerner, R. 1996. On signal sequence polymorphisms and diseases of distribution. *Proc Natl Acad Sci.*, 93:4471-4473.

Russo, J. Hu, Y. F. Yang, X. Russo, I. H. 2000. Developmental cellular, and molecular basis of human breast cancer. *J Natal Cancer Inst Monogr,* 27 : 17-37.

Shimoda-Matsubayashi, S. Matsumine, H. Kobayashi, T. Nakagawa-Hattori, Y. Shimizu, Y. and Mizuno, Y. 1996. Structural dimorphism in the mitochondrial targeting sequence in the human manganese superoxide dismutase gene. A predictive evidence for conformational change to influence mitochondrial transport and a study of allelic association in Parkinson's disease. *Biochem. Biophys. Res. Commun*., 226: 561–565.

Sutton, A. Khoury, H. Prip-Buus, C. Cepanec, C. Pessayre, D. and Degoul, F. 2003. The Ala16Val genetic dimorphism modulates the import of human manganese superoxide dismutase into rat liver mitochondria. *Pharmacogenetics*, 13:145-157.

Tamimi, R. M. Hankinson, S. E. Spiegelman, D. Colditz, G. A. and Hunter, D. J. 2004. Manganese superoxide dismutase polymorphism, plasma antioxidants, cigarette smoking, and risk of breast cancer. *Cancer Epidemiol Biomarkers Prev.,* 13(6):989-996.

Wang, L. I. Miller, D. P. Sai, Y. Liu, G. Su, L. Wain, J. C. Lynch, T. J., and Christiani D. C. 2001. Manganese superoxide dismutase alanine-to-valine polymorphism at codon 16 and lung cancer risk. *J Natl Cancer Inst*., 93:1818–1821.

Waris, G. and Ahsan, H. 2006. Reactive oxygen species: role in the development of cancer and various chronic conditions. *Journal of Carcinogenesis*, 5:14-21.

World Health Organization, Fact sheet No. 297 "Cancer", 2006.

JJBS

Jordan Journal of Biological Sciences

Volume 2, Number 2, June 2009
ISSN 1995-6673
Pages 83 - 88

Predicting Species Relative Abundance in Ecological Communities

Nidal Odat[a,*], Moh'd T. Moh'd Taleb Alodat [b], Riyadh Muhaidat[c], Faisal Ababneh[d], Abdel Rahman M. Al-Tawaha[a], Salem Aladaileh[a]

[a]*Department of Biological Sciences, Al-Hussein Bin Talal University, Jordan*

[b]*Department of Statistics,* [c]*Department of Biological Sciences, Yarmouk University, Jordan*

[d]*Department of Mathematics and Statistics, Al-Hussein Bin Talal University, Jordan*

Abstract

Ecologists are increasingly appreciating using statistical models to predict aspects of species ecology including their abundance and distribution due to their importance in biological conservation and management practices. The aim of this study is to propose a statistical model that allows predicting previously unknown plant species relative abundance (SRA) in an unsurveyed region based on small sub-samples of the whole community. We apply the model to a biodiversity data set which includes plant relative abundances collected from sub-samples of varied communities in central Europe. The results show that the predicted plant relative abundances in unsurveyed sites are close in value to those in the known sites, reflecting the accuracy and the predictive power of the model in estimating species relative abundance in previously unsurveyd ecological sites. The importance of our model is discussed in relation to conservation biology and management.

الملخص

غالبا ما يوظف باحثو علم البيئة نماذج احصائية تمكنهم من التنبؤ عن مفاهيم تتعلق ببيئة الكائنات الحية بما فيها الوفرة والانتشار وذلك نظرا لأهمية هذه المفاهيم في الحفاظ الحيوي والممارسات الإدارية المرافقة لها. تهدف هذه الدراسة إلى اقتراح نموذجا إحصائيا يسمح بالتنبؤ عن الوفرة النسبية الغير معروفه أصلا لأنواع نباتية معينة في منطقة غير مدروسة سابقا وذلك بالاعتماد على عينات مبسطة وممثلة لكافة أفراد المجتمع النباتي في تلك المنطقة. لقد قمنا بتطبيق هذا النموذج الإحصائي على مجموعة من البيانات تتعلق بالتنوع النباتي والتي تم جمعها من مجتمعات نباتية في أوروبا الوسطى. أظهرت نتائج الدراسة وجود تقارب بين قيم الوفرات النسبية المتنبأ عنها في المواقع الغير مدروسة وتلك القيم المحصلة في المواقع المعروفة. في النهاية, تمت مناقشة أهمية هذا النموذج الإحصائي من خلال علاقته بالحفاظ الحيوي والممارسات الإدارية.

Keywords: Species Relative Abundance; Statistical Model, Biodiversity; Ecological Communities; Conservation Biology.

1. Introduction

The species relative abundance, typically estimated by calculating the abundance of a given species divided by the total abundances of all other species in an ecological community, is a fundamental description of an ecological community (Ricklefs and Schluter 1993; Gaston and Spicer, 1998). Although estimating the species relative abundance is basic, yet it represents informative data for ecologists and conservation biologists (Gatson 2003).

Ecologists use data of species relative abundance to infer information about the mode of interaction and type of relationship among different species in a given community (Ferrier and Guisan, 2006). Moreover, species relative abundances data is used to perform population viability analysis (Possingham, *et al*., 2001), and estimate ecological disturbance (Regan, *et al*., 2003). In conservation biology and management, information on relative abundances is of great importance, as for example, to study the impact of habitat disturbances, such as fragmentation. It is well known that that disturbed and fragmented habitats are usually dominated by a very few species compared to the undisturbed sites (Guisan, *et al*. 1999; Regan *et al*., 2003; IUCN, 2001; Guisan and Thuiller, 2005).

Ecologists are often interested in employing statistical models to predict the occurrence and the distribution of species due to their importance in conservation biology of species (Franklin, 1995; Guisan and Zimmermann, 2000; Ferrier, 2002; Zhang, 2007). Generally, these statistical models utilize correlations between data on species occurrences, relative abundances, and environmental predictors (Franklin, 1995).

In the present study, we develop a statistical model that allows predicting species relative abundance across ecological communities. However, the model requires data

* Corresponding author. odatnidal@hotmail.com.

about the relative abundance of sub-samples collected from a given community. Predicting species relative abundance in unsurveyed communities is extremely likely, given a data set of species relative abundances of representative sub-samples from various communities is available. We apply the model to field data of plant species relative abundances collected in a biodiversity project in central Europe in which plant species diversity and plant productivity have been investigated (Perner, *et al*., 2005). Finally, we discuss the importance of the model in fields of management and conservation biology

2. MATERIALS and METHODS

2.1. Data

The data used in the presented model was based on a biodiversity project carried out in a plateau-like mountain range at the Thuringian/Bavarian border in central Germany with a maximum height of 870 m. Average annual temperature in the area varies between 68F and 78F and average annual precipitation varies between 950 and 1099 mm (Perner, *et al*, 2005). The studied plant communities located between 11.018º and 11.638º eastern longitudes and between 50.358º and 50.578º northern latitudes, and were covering about one hectare. For the model we developed ten plant species were chosen, namely *Dactylis glomerata, Taraxacum officinale, Trifolium repens, Veronica chamaedrys*, *Anthoxanthum odoratum, Rumex acetosa, Ranunculus acris, Holcus lanatus, Phleum pretense,* and *Campanula rotundifolia*. The species were collected from nine distinct sites that are almost comparable with regard to elevation, edaphic and climatic factors (Kahmen, *et al*., 2005).

2.2. The Proposed Model

The primary goal of our model is to predict previously undetermined relative abundance of ten different plant species in unsurveyed site. Our prediction was based on known data collected from nine different sites within each community. For the model parameters consider biological communities with a large number of species. Let $X_1,\cdots,X_n$ be the species relative abundances drawn from a random sample of n size collected from a given community (Figure 1). A species relative abundance is a description of the abundance (number of individuals observed) for each species encountered within a community. As such, it is one of the most basic descriptions of an ecological Community (Ricklefs and Schluter, 1993; Gaston and Spicer, 1998). We assume that the probability density function which represents that community is $f(x\mid\theta)$, where θ a parameter of interest is. The Bayesian statistics use the sample information in addition to our prior knowledge about θ to make statistical inferences about the population. The prior knowledge about θ is given as a probability distribution $\pi(\theta)$, say. We assume that the relative abundances for species community follow the power function density, i.e.,

$$f(x\mid\theta)=\begin{cases}\dfrac{\theta}{1-\theta}x^{\frac{2\theta-1}{1-\theta}}, & 0<x<1,\,0<\theta<1;\\ 0 & otherwise,\end{cases}$$

where θ represents the mean of the distribution,.i.e., θ is the mean of relative abundances in whole community. As a prior knowledge about θ, we assume that $\pi(\theta)=\theta^{-1}(1-\theta)^{-1}$, the Jeffery's no-informative prior. Using both sources of information, we update the density to the posterior density

$$\pi(\theta\mid X_1,\cdots,X_n)=cf(X_1\mid\theta)\cdots f(X_n\mid\theta)\pi(\theta),$$

where c is a normalizing constant. If Y is the relative abundance of an un surveyed site in this community, then we can predict Y according to the predictive density

The mean and the variance of the density $f(y\mid X_1,\cdots,X_n)$ are the prediction of Y and its uncertainty. Combining the power function observations together with the prior information leads us to the following predictive density :

$$f(y\mid t)=\frac{\int_0^1\theta^n(1-\theta)^{-n-2}\exp(-\frac{\theta t}{1-\theta}+\frac{2\theta-1}{1-\theta}\log y)d\theta}{\Gamma(n)t^{-n}},$$

$$=\frac{nt^n}{y(t-\log y)^{n+1}};\qquad \text{for } 0<y<1$$

where $t=-\sum_{i=1}^{n}\log X_i$.

The mean of this density is

$$\hat{Y}=\int_0^1\frac{nt^n}{(t-\log y)^{n+1}}dy; \tag{1}$$

and the prediction error =

$$\int_0^1\frac{nt^n y}{(t-\log y)^{n+1}}dy-\hat{Y} \tag{2}$$

All calculation and mathematical formula have been carried out with the help of the software package MATHEMATICA.

3. Results and Discussion

The model in this study is based on a biodiversity study conducted in Central Europe (Germany) in which plant species diversity, productivity and site characteristics have been investigated using multivariate statistics. In each study site a 2x2 m plot was established and used to determined plant cover, community biomass (productivity), and soil nutrients. The plant species within the 2x2 m plot were identified to the species level and the percent cover of each species was estimated visually (Perner, et al., 2005). This data was then used to calculate the relative abundance for each plant species as the number of plant of a particular species as a percentage of the total number of all plants of a given area or community. The plant communities in the study sites include all associated plant species that form the natural vegetation at each geographical site (Gaston, 2003).

The model provides a statistical tool for predicting species relative abundance at a given community. Table 1 shows the predictive relative abundances for the ten plant

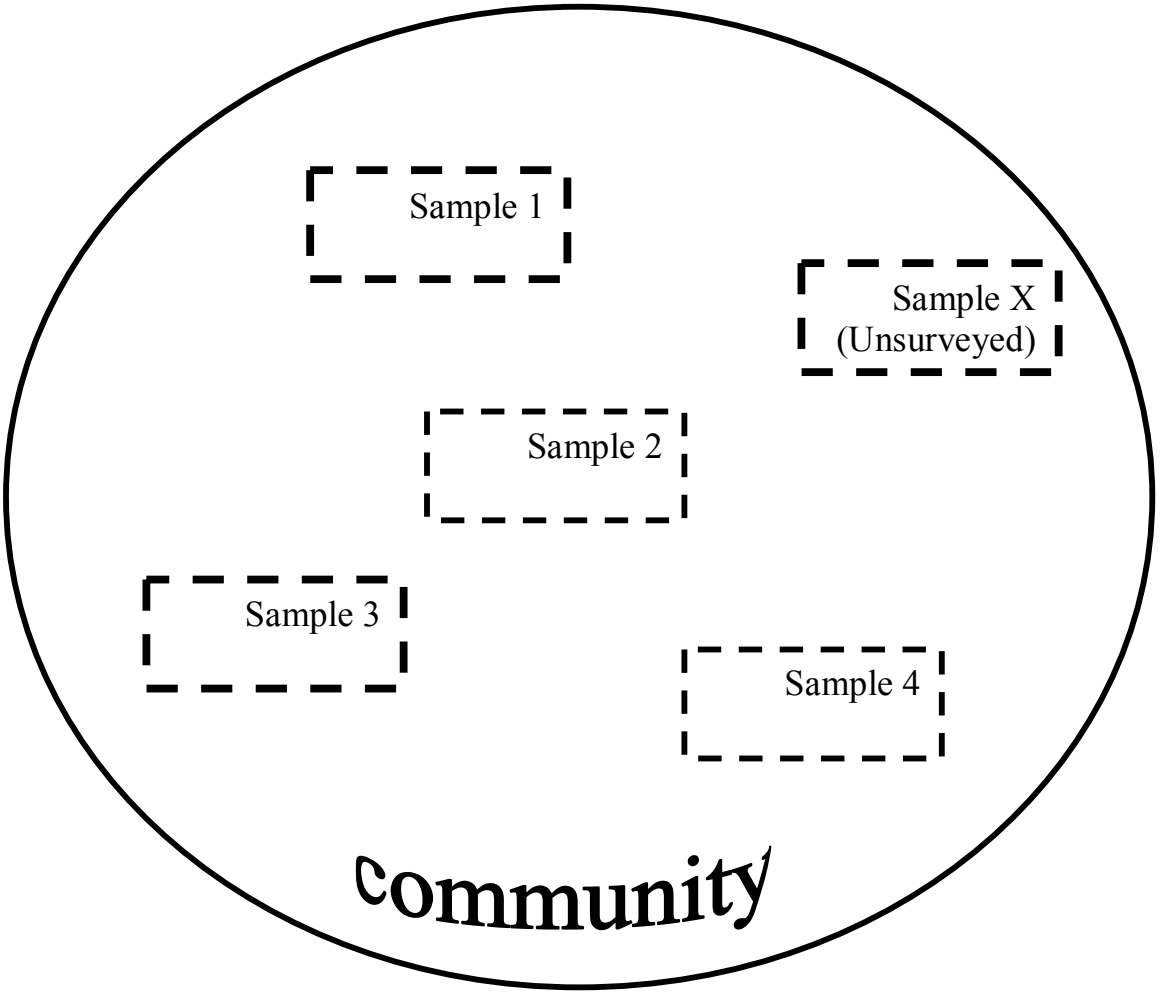

Figure 1. Hypothetical community with four known sub-samples (sample 1-4) and one unsuveyed sample.

Table 1. The predictive relative abundances for the ten plant species used in the model and their prediction error values. The prediction error was calculated according to equation 2 (see text) with the help of the software MATHEMATICA.

Plant species	Predicted relative abundances	Prediction error
Dactylis glomerata	0.252	0.081
Taraxacum officinale	0.221	0.076
Trifolium repens	0.216	0.075
Veronica chamaedrys	0.197	0.071
Anthoxanthum odoratum	0.273	0.085
Rumex acetosa	0.203	0.073
Ranunculus acris	0.153	0.060
Holcus lanatus	0.209	0.074
Phleum pretense	0.174	0.066
Campanula rotundifolia	0.150	0.059

species that are most common in the studied communities: (Dactylis glomerata, Taraxacum officinale, Trifolium repens, Veronica chamaedrys, Anthoxanthum odoratum, Rumex acetosa, Ranunculus acris, Holcus lanatus, Phleum pretense, and Campanula rotundifolia) which have been investigated in the present paper. The results show that the relative abundances for the species in the unsurveyed site are close in value to those in the known sites, reflecting the accuracy of our model in estimating species relative abundance ecological communities. Relative abundance and frequency of the predicted value for each plant species and for the whole plant community from which each species has been sampled are illustrated in Figure 2. These results indicate that the predictive values for each species are located within the density of the relative abundance calculated from the field data which may be suggest that our model is informative and comprehensive. To test the validity of the model we used simulation test to manipulate the relative abundance of five plant species drawn randomly from each community and compared the results of simulation with those obtained by our model. The results of the simulation

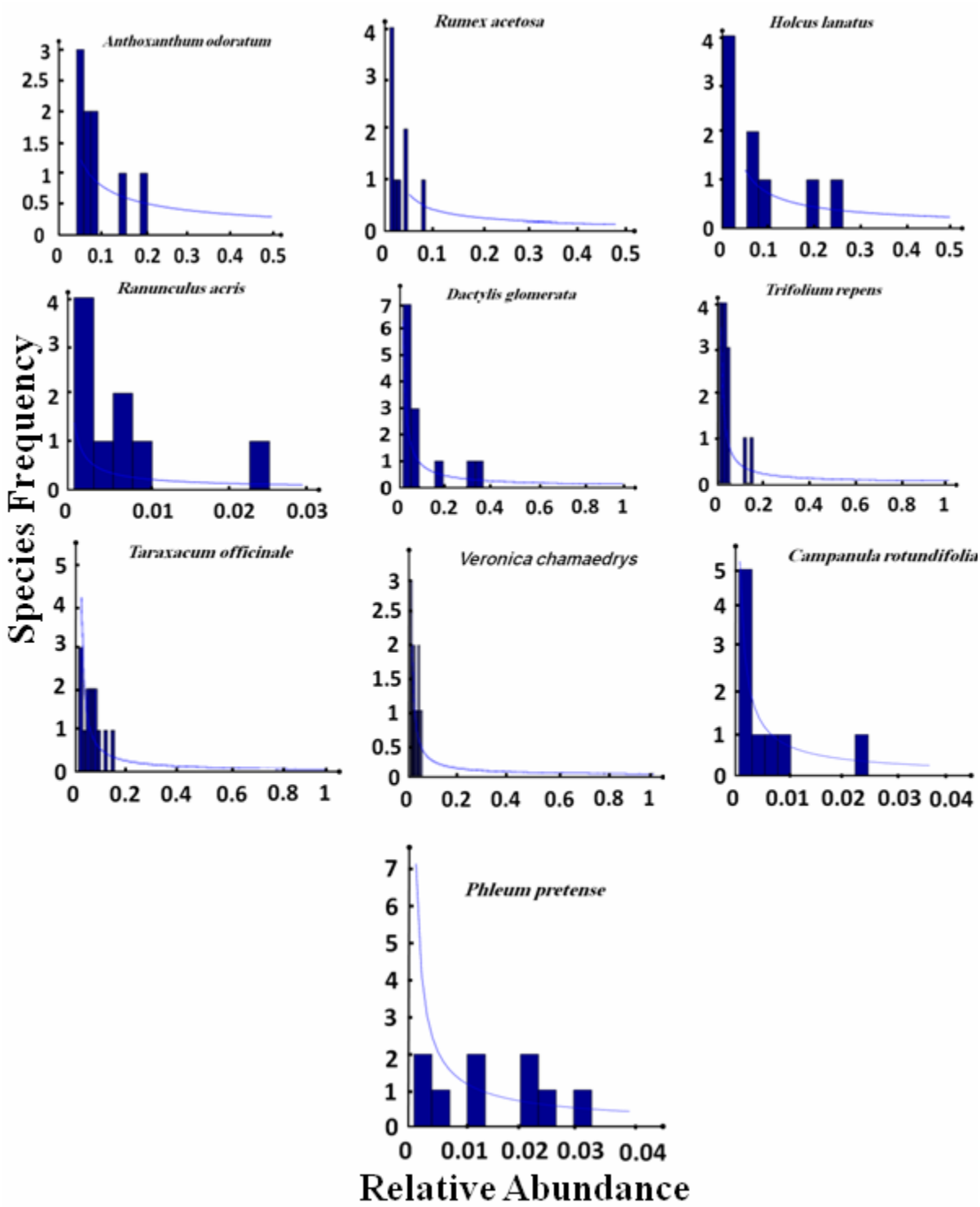

Figure 2. Histograms of the relative abundances for the ten plant species (Dactylis glomerata, Taraxacum officinale, Trifolium repens, Veronica chamaedrys, Anthoxanthum odoratum, Rumex acetosa, Ranunculus acris, Holcus lanatus, Phleum pretense, and Campanula rotundifolia). Lines are the theoretical density which represents the species relative abundance in the whole plant community.

includes two estimates for the relative abundance of each species (one estimate is based on the proposed model and one based on the simulation test). The simulation results reveal that the two values of each estimate are very close reflecting the predicated power of the model (Table 2). We propose that this model may have a wide range of applications, particularly in management practices and biological conservation. For instance, information on species relative abundance at a certain habitat can help us judge if that habitat is under human impact, such as disturbance and fragmentation. It is well known that disturbed habitats are usually dominated by a very few species compared to the undisturbed, more diverse sites (Guisan, *et al*., 1999; Guisan and Zimmermann, 2000; Regan, *et al*., 2003; Guisan and Thuiller, 2005).

The model presented in this study is rather simple and limited to habitats of similar ecological conditions, in term of species composition and abiotic factors. Nevertheless,

Table 2. The species relative abundances of five selected plant species as derived from the model and from the simulation test.

Plant species	Relative abundance based on model	Relative abundance based on simulation
Anthoxanthum odoratum	0.2796	0.2791
Rumex acetosa	0.2071	0.2062
Ranunculus acris	0.1532	0.1587
Holcus lanatus	0.2132	0.2044
Phleum pretense	0.2031	0.1820

our results maybe suggest that statistical models open the door for testing a variety of hypotheses regarding species relative abundances of plant species. The model established here can further be employed to settle other fundamental research questions related specifically to effects of population structure, species spatial distribution pattern, and habitat patchiness (homogenous vs. heterogeneous) (Lichstein, *et al.*, 2002; Holt, 2003; Reese, *et al*., 2005; Hoeting, *et al*., 2006).

References

[1] Ferrier S. 2002. Mapping spatial pattern in biodiversity for regional conservation planning: where to from here? Sys. Biol. 51: 331–363.

[2] Ferrier S and Guisan A. 2006. Spatial modeling of biodiversity at the community level. J. Appl. Ecol. 43: 393–404.

[3] Franklin J. 1995. Predictive vegetation mapping: geographic modelling of biospatial patterns in relation to environmental gradients. Prog. Phys. Geog. 19: 474–499.

[4] Gaston K. 2003. The structure and dynamics of geographic ranges. Oxford University Press, Oxford.

[5] Gaston KJ and Spicer VI. 1998. Biodiversity: an introduction. Blackwell Science, Oxford, UK.

[6] Guisan A and Zimmermann NE. 2000. Predictive habitat distribution models in ecology. Ecol. Model. 135: 147–186.

[7] [7] Guisan A and Thuiller W. 2005. Predicting species distribution: offering more than simple habitat models? Ecol. Lett. 8: 993–1009.

[8] Guisan A, Weiss SB and Weiss AD.1999. GLM versus CCA spatial modeling of plant species distribution. Plant. Ecol. 143: 107–122.

[9] Hoeting JA, Davis RA, Merton AA and Thompson SE. 2006. Model selection for geostatistical models. Ecol. Appl. 16: 87–98.

[10] Holt RD. 2003. On the evolutionary ecology of species' ranges. Evol. Ecol. Res. 5: 159–178.

[11] IUCN (2001) The IUCN Red List of Threatened Species. 3.1, Gland, Switzerland.

[12] Kahmen A, Perner J, Audorff J, Weisser W and Buchmann N. 2005. Effects of plant diversity, community composition and environmental parameters on productivity in semi-natural montane European grasslands. Oecologia 142: 606-615.

[13] Lichstein, JW, Simons TR, Shriner SA, and Franzreb KE. 2002. Spatial autocorrelation and autoregressive models in ecology. Ecol. Monogr. 72(3):445-463.

[14] Perner J, Wytrykush C, Kahmen A, Buchmann N, Egerer I, Creutzburg S, Odat N, Audorff V and Weisser WW. 2005. Effects of plant diversity, plant productivity and habitat parameters on arthropod abundance in montane European grasslands. Ecography 28: 429–442.

[15] Possingham HP, Lindenmayer DB and McCarthy MA. 2001. Population Viability Analysis, Encyclopædia of Biodiversity. Academic Press, pp 831– 843.

[16] Reese GC, Wilson KR, Hoeting JA and Flather CH. 2005. Factors affecting species distribution predictions: a simulation modeling experiment. Ecol. Appl. 15: 554–564.

[17] Regan HM, Auld TD, Keith DA and Burgman M.A. 2003. The effects of fire and predators on the long-term persistence of an endangered shrub, *Grevillea caleyi*. Biol. Conserv. 109: 73–83.

[18] Ricklefs RE and Schluter D. 1993. Species diversity in ecological communities: historical and geographical perspectives. University of Chicago Press, Chicago, Illinois, U.S.A

[19] Zhang H. 2007. Inferences on the Number of Unseen Species and the Number of Abundant/Rare Species. J. Appl. Statist. 34: 725–740.

JJBS

Jordan Journal of Biological Sciences

Volume 2, Number 2, June 2009
ISSN 1995-6673
Pages 89 - 96

Genetic Relatedness among Wild and Cultivated Almond Genotypes Using Randomly Amplified Polymorphic DNA (RAPD) Markers in Jordan

Abdul Latief Ali Al-Ghzawi[a], Ibrahim M Rawashdeh[b,*], Abdel Rahman Al-Tawaha[c]

[a]*The Hashemite University, Dept. of Biology and Biotechnology, Zarqa , Jordan*

[b]*National Center for Agricultural Research and Extension (NCARE), Baq'a, Jordan*

[c]*Al Hussein Bin Talal University, Department of Biological Sciences, Ma'an, Jordan*

Abstract

Randomly amplified polymorphic DNA (RAPD) technique was used to study the genetic relatedness between 16 almond cultivars (*Prunus. Dulcis)* grown at field gene bank (Al-M'shaqer Research Station), wild genotypes and farmers' orchards genotypes in Jordan. Five primers showed polymorphic bands were used for construction dendrogram and similarity matrix. Similarity values among the studied genotypes were ranged from 0.000 to 0.500. High similarity values were obtained between the two sweet almond cultivars Fuksii (0.500) and Tuono (0.480). RAPD analysis confirmed the existence of genetic variation among tested almond cultivars. Oja cultivar was the most distant compared to the rest of the cultivars, even, wild types and sweet genotypes were grouped separately. Using a minimum number of samples with the most polymorphic RAPD primers was a key for having rapid results of genetic relationships among almond genotypes.

الملخص

استخدمت تقنيةRAPD لدراسة الارتباط الوراثي بين 16 صنف لوز المزروع في الحقل الوراثي (محطة المشقر) والأنواع البرية والمزروعة في حقول المزارعين. خمسة بادئات أظهرت باندات متشعبة استخدمت لبناء الشجرة العنقودية والتشابه.قيم التشابه بين الأنواع المدروسة كانت بين صفر- 50%. أعلى قيم التشابه كانت بين نوعين من صنف اللوز فوسكي (50%) وتونو 48% . إن التحليل RAPD اثبت وجود اختلافات وراثية بين الأنواع المدروسة. صنف عوجا كان أكثر بعدا وراثيا مقارنة الأصناف الأخرى وكانت الأنواع البرية والأنواع الحلوة تجمعت في مجموعات مفصولة عن بعضهم. إن استخدام عدد قليل من العينات مع أكثر البادئات المتشعبة تعبر المفتاح للحصول على نتائج للعلاقات الوراثية بين أنواع اللوز.

Keywords: Almond; Jordan; Polymorphism; *Prunus; Dulcis;* RAPD; Wild.

1. Introduction

Almond [*Prunus dulcis* (Mill.) D. A. Webb; syn. *P. amygdalus* Batsch] which belongs to the subgenus *Amygdalus* (Rosaceae, subfamily Prunoideae) (Martínez – Gómez *et al.*, 2007), is characterized by its adaptability to arid and semi arid regions. Almonds have high nutritive value, high lipid content with concentrated energy sources and can be used in many food products. (Ahrens *et al*., 2005; Martínez –Gómez *et al*., 2007), and it is widely grown at the Mediterranean area including Jordan, Syria, Turkey and Iraq. (Martins et al. 2004; Martínez –Gómez et al., 2007).

In Jordan, wild almonds (with small nuts, hard-shelled and bitter kernels) were grown at the high mountains. Kester *et al.* (1991) reported that the native almond species predominantly have bitter kernels because of high levels of the glucoside amygdalin, and the forest department at agriculture ministry of Jordan was adopted the planting this type in order to become the source of seeds as well to cover the lands and protect the soil from erosion. Moreover, it is used as a rootstock for sweet almond and peach due to it's high adaptability to drought and disease resistance. Although, large numbers of almond native genotypes in Jordan show attractive characteristics such as color type, but most of the sweet cultivars were introduced from other countries. Twenty four cultivars were planted in Jordan, (during 1996), at Al-M'shaqer Research Station in the field gene bank for research purposes (Al-Hmoud *et al*., 2006).

* Corresponding author. irawashdeh2002 @yahoo.com.

Traditionally, the identification and characterization of almond cultivars was based on morphological traits. However, such traits were not always available for analysis because of it's highly environmental effect, and it may only be visible in adult materials (Martínez–Gómez *et al*., 2007). In Lebanon, Talhouk *et al.* (2000) studied the phenotypic diversity and morphological characterization of *Amygdalus* species and found high genetic diversity of *Amygdalus communis* L., *Amygdalus korshinskyi* Hand.-Mazz., and *Amygdalus orientalis* Duh.

Molecular DNA markers have succeeded in distinguishing among accessions, clarifying synonyms, identifying mislabeled cultivars, establishing genetic similarities or geographical origins and giving hints about the process of domestication (Wünsch and Hormaza, 2002). Morphological and biochemical studies were unable to emphasis the relatedness due to their exposure to environmental influences. Therefore, the molecular marker techniques have a great potential for studying the genetic variation and relatedness among targeted cultivars. Among those markers, RAPD has the ability to detect the genetic variation among numerous of plants and animals species. The main advantages of RAPD are: adequate for a primer screening of a large number of samples, rapid scanning of the genome, higher band-sharing, greater number of loci per assay and less laborious test (Bara´nek et al., 2006 and Vidal *et al*., 1999). Wünsch and Hormaza, (2002) mentioned that RAPD markers were used by several laboratories to identify genotypes of different temperate fruit tree species.

MirAli and Nabulsi, (2003) and Shiran et al. (2007) used RAPD technique to study the genetic relatedness among Syrian and Iranian almond cultivars, respectively. Moreover, Gouta *et al*. (2007) used RAPD technique and reported that the similarity values among 58 almond cultivars were ranged from 0.45 to 0.94. RAPD markers have been used to characterize *Prunus* rootstocks from different species (Casas *et al*., 1999) such as studying the genetic relationship among grapevine varieties (Vidal *et al*., 1999) and determining the diversity level among 24 Iranian pomegranate genotypes (Sarkhosh *et al*., 2006).

Since no molecular information is available regarding almond cultivars grown in Jordan. This study aimed at determining the genetic relationships among 16 almond cultivars grown in the field gene bank at Al-M'shaqer Research Station, farmers' orchards genotypes and wild type genotypes using RAPD technique. The results of this study will be used for monitoring, management and conservation of almond types in the future.

2. Materials and Methods

2.1. Plant Material

Fresh leaves were collected from 16 almond cultivars grown at the field gene bank of Al-M'shaqer Research Station in Jordan, 23 genotype of farmers' orchards, 4 unknown and 12 wild types (Table 1).

2.2. DNA Isolation

DNA was obtained after grounding the stored leaves (4ºC) of almond samples in liquid nitrogen (-196ºC), the quality and quantity of genomic DNA were detected on agarose gel. Total cellular DNA was extracted, using procedure as described by (Doyle and Doyle, 1987), with minor modifications of chemical concentrations. Approximately 18 to 20 mg of fresh and stored leaves were ground and mixed with 750 µl of freshly and preheated 2x CTAB solution with 0.8g PVPP in 2ml tubes then placed at 65ºC for 30 min. The mixture was incorporated with 750 µl of chlorophorm/isoamyl alcohol (24:1), vortexed for few seconds, and then centrifuged at 14,000g for 20 min. The supernatant was placed in 2ml tubes with 600ml isopropanol, and then shaked until the thread of DNA was appeared, then centrifuged for 20 min at 14000g. The solution is poured in tubes and left to dry, then 600 µl of cooled 70% ethanol was added to the solution and placed in the refrigerator (–20ºC) overnight. Next day, ethanol was poured in the dried tubes and 100µl of TE was added and the whole mixture was placed at 65ºC for 30min. Four microlitters of RNase (10mg/ml) were added per tube and left for 45min at 37ºC. DNA quantity was measured using S2100 UV/VIS DIODE-Array-Spectrophotometer, machine Version 1.7.

2.3. PCR Amplification

PCR reaction was performed as described by Williams *et al.* (1990) with 10-mer ologonucleotides synthesized by Opern technologies (Almeda, Calif.). The final volume of 25µl containing, 10x buffer, 20ng of total genomic DNA, 10 mM dNTPs, 100 µM of primers, 1.5Mm MgCl2 and 1U of *Taq* polymerase. Amplification was carried out in thermocycler (MJ Research model PCT-100), one cycle of 1 min at 94°C followed by 44 cycles, each consisting of a denaturation step for 1min at 94°C, followed by an annealing step for 1min at 36°C and an extension step for 2 min at 72°C, followed by a further extension step for 5 min at 72°C. After the final cycle the samples were cooled at 4°C. Samples of 10 µl were analyzed by electrophoresis on 1.4% agarose gel and the amplified products were detected after staining by ethidium bromide.

The RAPD-PCR product was detected in 1.4% agarose mixed with 0.5X TBE. Forty 10-mer primers, corresponding to kits A, B, C, N and Z by Operon Biotechnologies, were initially applied to the whole group of genotypes. Each primer was applied twice. Only repeatable fragments with strong and medium intensity were evaluated.

2.4. Data Analysis

RAPD polymorphic bands were scored as present (1) or absent (0) and the estimation of similarity among all accessions were calculated according to Ne and Li, (1979). The matrix of similarity was analyzed by the Unweighted Pair-Group Method (UPGMA) and the dendrogram was obtained using SPSS, V. (11.0), software to estimate genetic similarities with the Jaccard's coefficient.

3. Results

From 40 initially applied primers, only 6 showed reproducible fragments with easily recordable fingerprints. When screening all the 16 cultivars, 4 unknown genotypes, 16 sweet genotypes, 12 wild and (3 Awajee, one awajee,

Table 1. List of almond cultivars, wild genotypes and frames orchard genotypes grown in Jordan.

Type/ name	Source	Type/ name	Source
1-Wild	Wadi Shouiab /Sult/ farmer orchard	29-Wild	Alaal- Irbid / farmer orchard
2-Wild	Wadi Shouiab /Sult/ farmer orchard	30-Awajee	Samarwsan/ farmer orchard
3-Sweet*	Wadi Shouiab /Sult/ open fields	31-Sweet	Samarwsan/ farmer orchard
4-Sweet	Wadi Shouiab /Sult/ open fields	32-Sweet	Samarwsan/ farmer orchard
5-Sweet	Wadi Shouiab /Sult/ open fields	33-Sweet	Samarwsan/ farmer orchard
6-Sweet	Wadi Shouiab /Sult/ open fields	34-Sweet	Samarwsan/ farmer orchard
7-Sweet	Wadi Shouiab /Sult/ open fields	35-Sweet	Samarwsan/ farmer orchard
8-Flakee	Ajloun/ farmer orchard	36-Sweet	Samarwsan/ farmer orchard
9-Wild	Ajloun/ farmer orchard	37-Sweet	Samarwsan/ farmer orchard
10-Wild	Ajloun/ farmer orchard	38-Sweet	Samarwsan/ farmer orchard
11-Wild	Ajloun/ farmer orchard	39-Sweet	Samarwsan/ farmer orchard
12-Wild	Ajloun/ farmer orchard	40-Hamah	Field gene bank Al-M'shaqer Research Station /NCARE
13-Unknown	Sakhrah / Ajloun/ farmer orchard	41-Primorski	Field gene bank Al-M'shaqer Research Station /NCARE
14-Unknown	Sakhrah / Ajloun/ farmer orchard	42-Douma 1	Field gene bank Al-M'shaqer Research Station /NCARE
15-Unknown	Sakhrah / Ajloun/ farmer orchard	43-Chellaston	Field gene bank Al-M'shaqer Research Station /NCARE
16-Unknown	Jerash/ Balilah/forest reserve	44-Douma 3	Field gene bank Al-M'shaqer Research Station /NCARE
17-Wild	Jerash/ Balilah/ forest reserve	45-Ne Plus Ultra	Field gene bank Al-M'shaqer Research Station /NCARE
18-Wild	Jerash/ Balilah/ forest reserve	46-SF121	Field gene bank Al-M'shaqer Research Station /NCARE
19-Wild	Jerash/ Balilah/ forest reserve	47- Shami fark	Field gene bank Al-M'shaqer Research Station /NCARE
20-Wild	Jerash/ Balilah/ forest reserve	48- Princesses	Field gene bank Al-M'shaqer Research Station /NCARE
21-Wild	Jerash/ Balilah /forest reserve	49-Ardshwar	Field gene bank Al-M'shaqer Research Station /NCARE
22-Shami	Koufrawn/ farmer orchard	50- Ardoma	Field gene bank Al-M'shaqer Research Station /NCARE
23-Awjah	Koufrawn/ farmer orchard	51-Dafadii	Field gene bank Al-M'shaqer Research Station /NCARE
24-Fark	koufrawn/ farmer orchard	52-Texas	Field gene bank Al-M'shaqer Research Station /NCARE
25-Awajee	Alaal- Irbid/ farmer orchard	53-Fuksii	Field gene bank Al-M'shaqer Research Station /NCARE
26-Awajee	Alaal- Irbid/ farmer orchard	54-Tuono	Field gene bank Al-M'shaqer Research Station /NCARE
27-Sweet	Alaal- Irbid/ farmer orchard	55-Oja	Field gene bank Al-M'shaqer Research Station /NCARE
28-Sweet	Alaal- Irbid/ farmer orchard	----------	----------

* local name

Table 2. Statistical reading of RAPD analysis.

Primer name	Total bands/primer	Number of polymorphic bands	% of polymorphism	Max./ Min. band per primer
OPA05	119	35	29	3/10
OPA17	133	32	24	1/8
OPA20	293	45	15	2/9
OPN14	174	25	14	1/7
OPN16	261	39	15	1/9
Total band	980	Average : 35.2	Mean: 19.4	

one Flakii, one Fark and one Shami cultivars, from farmers orchids) five primers (12.5% of primers were polymorphic) shown polymorphism in the whole group analysis. Total number of bands, number of polymorphic bands and percent of polymorphism and maximum/minimum number of bands per primer were depicted by Table 2. A total of 980 RAPD fragments (Table 2) and 176 bands of them were polymorphic in the whole genotypes group and generating 14.2% polymorphism. Among the used primers, OPA05, OPA17, OPA20, OPN14 and OPN16 with recognized fingerprints showed the best suitability for identification, management, conservation and genetic study of almond species. Representative amplification patterns resultant from primer OPA20 and OPN16 shows at Figure 1 and Figure 2.

The number of bands detected by each primer depends on primer, sequence and the extent of variation in specific genotype (Shiran *et al.*, 2007). Therefore, the number of bands varied in different genotypes. In this study, number of bands varied from one to ten bands with an average of 8 bands.

High levels of similarity ranged between 0.500 to 0.000 (Table 3). All almond cultivars were the most distant from

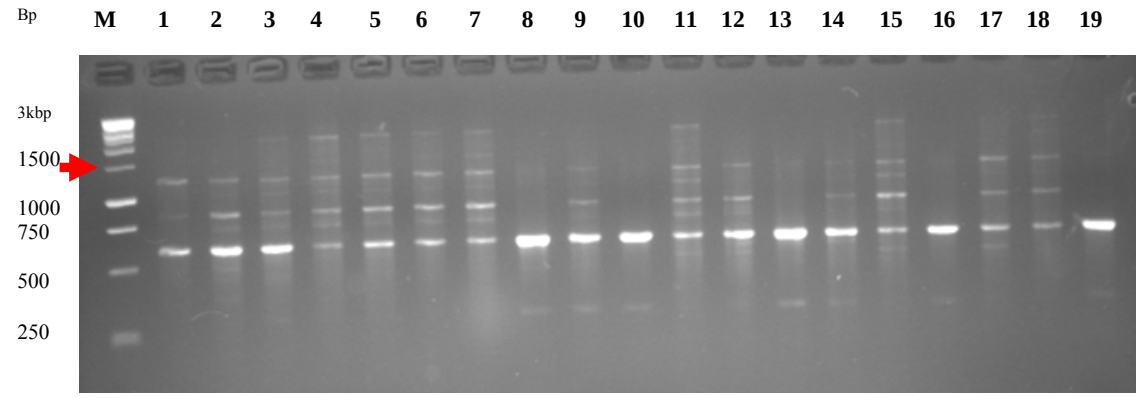

Figure 1. PCR-RAPD profiles generated from different genotypes of almond using primer **OPA20**. Lane 1: Wild ; lane 2: Wild ; lane 3: Sweet ; lane 4: Sweet; lane 5: Sweet; lane 6 : Sweet; lane 7: Sweet; lane 8: Flakee; lane 9: Wild; lane10: Wild; lane11: Wild; lane12: Wild; lane 13: Unknown; lane 14: Unknown; lane 15: Wild; lane 16: Wild ; lane17: Wild; lane 18: Wild; lane 19: Wild; Lane M: 1kb ladder (Promega).

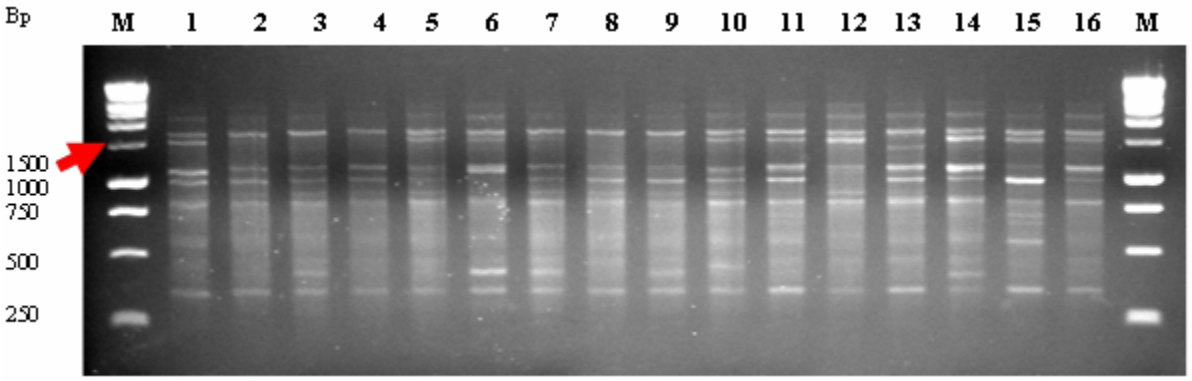

Figure 2. Agarose gel electrophoresis of DNA fragments amplified from different cultivars of almond using primer **OPN16**. Lane 1: Hamah; lane 2: Primorski; lane 3: Douma 1; lane 4: Chellaston; lane 5: Douma 3; lane 6: Ne Plus Ultra; lane 7: F121; lane 8: Shami fark; lane 9: Princesses; lane 10: Ardshwar; lane 11: Ardoma; lane 12: Dafadii; lane 13: Texas; lane 14: Fuksii; lane 15: Tuono; lane 16: Oja; Lane M: 1kb ladder (Promega).

Table 3. Highest and lowest similarity values among selected almond genotypes grown in Jordan.

Series No.	Type	Similarity value	Series No.	Type	Similarity value	Series No.	Type	Similarity value
1Wild	5 sweet 26 awajee 11 sweet	0.267 0.150 0.158	12Wild	15unknown 28sweet	0.304 0.280	23Awajee	25Awajee 27sweet 29wild 31sweet 7sweet	0.452 0.343 0.360 0.276 0.303
2- Wild	21 wild 34 sweet	0.278 0.206	13Unknown	16unknown 30awajee	0.412 0.222	24Fark	26Awajee 29wild 31sweet	0.419 0.379 0.280
3- Sweet	4 sweet 5 sweet 6 sweet 15unknown	0.333 0.269 0.280 0.292	14Unknown	17wild 18wild	0.429 0.200	25Awajee	29wild 33sweet 31sweet 45Ne Plus Ultra	0.438 0.429 0.31 0.000
4- Sweet	7sweet 33sweet 32sweet 29wild 5sweet	0.433 0.258 0.250 0.250 0.242	15Unknown	18wild 48Princesses 8 Flakee	0.292 0.000 0.241	26Awajee	27sweet 28sweet 29wild 24Fark	0.368 0.353 0.316 0.419
5 Sweet	11 sweet 37 sweet 33 sweet	0.222 0.189 0.200	16Unknown	31sweet 40Hamah 41Roma 44Douma3 45NePlusUltra 48Princesses 49Ardshwar	0.200 0.000 0.000 0.000 0.000 0.000 0.000	27Sweet	26Awajee 23Awajee 28sweet 27sweet	0.368 0.347 0.263 0.222
6 Sweet	7 sweet 12 wild 15 unknown	0.393 0.292 0.250	17Wild	14unknown 13unknown 18wild 43Chellaston	0.429 0.412 0.391 0.000	28 Sweet	24Fark 23awajee 26awajee 28sweet	0.379 0.364 0.353 0.200

				44Douma 3	0.000		31sweet	0.172
7 Sweet	33 sweet 23 Oja 25 awajee	0.310 0.303 0.257	18 Wild	19wild 40Hamah 43Chellaston 44Douma 3 17wild	0.333 0.000 0.000 0.000 0.391	29Wild	25awajee 31sweet 33sweet 43Chellas-ton	0.438 0.310 0.250 0.000
8 Flakee	16unknown 13unknown	0.333 0.238	19Wild	21wild 46SF121 51Dafadii 18wild	0.240 0.000 0.000 0.333	30Awajee	31sweet 10wild 36sweet 41primorski	0.333 0.286 0.227 0.000
9Wild	17wild 19wild 30Awajee	0.296 0.000 0.250	20 Wild	21wild 32sweet	0.154 0.135	31Sweet	30awajee 32sweet 43Chellas-ton 45NePlus Utra	0.333 0.320 0.000 0.000
10Wild	50Ardoma 30Awajee 31sweet 16unknown	0.000 0.286 0.208 0.208	21Wild	31sweet 27sweet	0.241 0.211	32Sweet	31sweet 33sweet 35sweet 45NePlus Ultra	0.320 0.296 0.292 0.000
11 Wild	17wild 26awajee	0.265 0.238	22Shami	24Fark 26Awajee	0.236 0.278	33Sweet	25awajee 7sweet 34sweet 35sweet 51Dafadii	0.429 0.310 0.269 0.154 0.000
Series No.	Type	Similarity value	Series No.	Type	Similarity value	Series No.	Type	Similarity value
34Sweet	35sweet 40Hamah 41Roma 43Chellst-on 45Ne Plus Ultra	0.318 0.000 0.000 0.000 0.000	42Douma 1	43Chellast. 44Douma3 47Shami 40Hamah	0.452 0.400 0.303 0.345	50Ardoma	51Dafadii 34sweet 53Fuksii 10 wild 13unknown	0.263 0.257 0.237 0.000 0.000
35Sweet	36sweet 40Hamh 41Rroma 45Ne Plus Ultra 47Shami 48Princesses 53Fuksii	0.227 0.000 0.000 0.000 0.000 0.000 0.000	43Chellaston	44Douma3 45Ne Plus 55Oja 12wild 17wild	0.286 0.229 0.225 0.000 0.000	51Dafadii	53Fuksii 52Dafadii 54Tuono	0.306 0.297 0.294
36Sweet	30awajee 41Roma 43Chellaston 44Douma3 50Ardoma 54Tuono	0.227 0.000 0.000 0.000 0.000 0.000	44Douma3	45NePlus Ultra 47ShamiFark 13 unknown 17 wild	0.333 0.265 0.000 0.000 0.000	52Texas	53Fuskii 54Tuono 55Oja 38sweet	0.424 0.375 0.324 0.000
37Sweet	38sweet 41Primorski 12wild	0.500 0.167 0.000	45NePlusUltr-a	46SF121 47ShamiFark 55Oja	0.250 0.242 0.257	53Fuskii	54Tuono 55Oja	0.483 0.297
38Sweet	43Chellaston 40Hamah 52Texas 12wild	0.189 0.000 0.000 0.000	46SF121	48Princesses 54Tuono 50Ardoma 47Shamifark	0.345 0.367 0.286 0.345	54Tuono	55Oja 10wild	0.250 0.000
39Sweet	42Douma 44Douma3 40Hamah 43Chellaston 3sweet 22Shami	0.324 0.286 0.273 0.263 0.000 0.000	47ShamiFurk	48Princesses 49Ardshwar 52Texas	0.345 0.227 0.215	55Oja	52Texas 53Fuskii 54Tuono	0.324 0.297 0.250
40Hamah	41Primorski 44Douma3 45Ne Plus Ultra 34sweet 12wild 17wild	0.462 0.393 0.345 0.000 0.000 0.000 0.000	48Princesses	51Dafadii 53Fuksii 52Texas 50Adoma 13unknown	0.333 0.323 0.313 0.236 0.000	--------	---------	---------
41Primorski	42Rouma 3 45Ne Plus Ultra 43Douma3 12 wild 16 unknown 22 Shami 34 sweet	0.414 0.345 0.294 0.000 0.000 0.000 0.000	49Ardshwar	50Ardoma 55Oja 52Texas	0.225 0.220 0.195	----------	----------	--------

the rest in the dendrogram. The highest average similarity index value among all varieties (0.48) was observed between Fuksii and Tuono. Most sweet genotypes showed high similarity values with each other (Table 3), with value more than 0.172. On the other hand, among 5 unknown genotypes, only two of them showed high similarity with sweet genotype (0.292 and 0.250) which could be confirmed that those are sweet genotypes, two registered 0.238 and 0.333 similarity values with Flakee variety, one unknown had 0.208 similarity with wild type. Sweet genotype showed high similarity values with Awajee genotype (0.429, 0.368, 0.347, 0.364, 0.353 and 0.438). Fark genotype had 0.419 similarity with Shami, Awajee showed 0.419 similarity with Awajee.

For almond cultivars, Hamah showed 0.462 similarities with Primorski and 0.000 similarity with sweet and wild genotypes, Primorski had 0.414 similarity with Douma 3 and 0.000 similarity value with Shami and sweet genotype. Douma 3 had 0.452 and 0.400 similarity with Chellaston and Douma 3, respectively, and 0.000 similarity with wild. Douma 3 has 0.333 similarity with Ne Plus Ultra. SF 121 had 0.367 with Touno. Shami Fark had 0.345 similarity with Princesses whereas, Princesses with Dafadii has 0.333 similarity value. Ardoma had 0.263 similarity with Dafadii whereas Dafadii had 0.306 similarity with Fuskii, but the former had 0.483 similarity with Fuskii. Oja had 0.324 similarity with Texas. However, there is no relationship between almond cultivars, wild and unknown genotypes. Based on the dendrogram, Oja was the most distant followed by one sweet genotype. Moreover, a dendrogram can be divided arbitrarily into three main clusters.

The first cluster contained, 4 unknown genotypes, 16 Sweet genotype, 11 wild, 2 Awajee, one Oja, one Flakii, one Fark, one Shami cultivar. This was divided into three sub-clusters, the first sub-cluster contained the most related genotypes (sweet-sweet genotype) with 0.500 similarity (Figure 3 and Table 3) two unknown, Flakii, Awajee, Sweet, Sweet, wild Sweet and Sweet). The second sub-cluster includes two unknown genotype, 5 wild genotypes and one sweet genotype. The third sub-cluster has 4 wild genotypes, one unknown, 10 sweet genotypes and the following cultivars including Oja, 2 Awajee, Fark and Shami.

The second major cluster contained only one wild genotype. The third major cluster contains 16 cultivars together with the most related cultivars (Tolono and Fuchsia). The third major cluster was divided into three sub-clusters, the first one contained Fuksii, Tuono, Texas, SF121, Princesses, Dafadii and Ardoma. While the second one includes Shami Fark and Redshawer and the third one contained Roma, Chellaston, Duma 3, Hamah, Primorski, Ne Plus Ultra, and Oja in addition to one sweet genotype.

4. Discussion

Knowledge of the genetic diversity and relationships among cultivated species of *Prunus* is important to recognize it is gene pool in order to identify pitfalls in germplasm collections, and to develop effective conservation and management strategies (Aradhya *et al*., 2004). Present study showed a high level of genetic variation existed in Jordanian almond gene pool as indicated by the wide range of similarity index values generated by using RAPD markers between cultivars and genotypes. These findings agreed with results obtained by Shiran et al. (2007). Presence of genetic variability among tested genotypes lead to the suitability of RAPD for determining genetic relationship either within cultivar or with other genotypes. The relatedness of the studied cultivars was efficient and good established through the use of RAPD markers.

Shiran *et al.* (2007) and Martinez-Gomez *et al*. (2003) used RAPD technique for almond and concluded that RAPD technique has the ability to discriminate between Sefied and Monagha almond cultivars. In the current study, wild types were clustered separately from other cultivars which mean that wild types were not ancestor of cultivated almonds and could be belong to other sources (rootstock); this result was supported by results obtained by Shiran *et al*. (2007). The lower similarity values and more divergence dendrogram branch points of wild species and cultivars demonstrate the greater genetic variability of these plant materials.

Wild almond species plays a socio-economical and ecological role as well they have been used for different purposes by native people including direct consume, grazing of livestock or oil extraction (Zeinalabedini *et al*., 2007). In this study, wild species which is usually considered as the origin of almond cultivars was found separately placed in one main cluster away from cultivars. Same results was observed by Shiran *et al*. (2007) who mentioned that the two wild species of almond (*P. orientalis* and *P. scoparia* were clustered out of the rest of cultivars and genotypes. The presence of wild crop relatives and the continuous flow of genes among and within plant species determined the rise of new genetic variability (Chessa and Nieddu, 2005). Considering the similarity matrixes values it is concluded that two varieties of Oja and Awajee were introduced from Syria are similar based on the similarity value of 0.452 (Table 3 and Figure 3). Because sweet genotypes were sub-grouped with awajee, it is concluded that all sweet genotypes could be Awajee with wild ancestor. Present study showed that almond genotypes or cultivars with similarity values less than 0.200 can be considered as genetically differs. However, Shiran *et al*. (2007) reported that the accessions of almond showing at least 20% genetic differences from each other may be regarded as distinct cultivars. The results obtained by RAPD indicated that this technique was efficient in detecting genetic similarities in almond. However, the results obtained from this study help in management, collection, monitoring and conservation of Jordanian almonds. Using techniques such as simple sequence repeats (SSR) and amplified fragment length polymorphic DNA (AFLP) in further investigation.

Acknowledgment

This research was funded by The Deanship of Scientific Research and Graduate Studies at The Hashemite University- Zarqa- Jordan. The authors would like to acknowledge the cooperation of the researchers of the NCARE who contributed to the work.

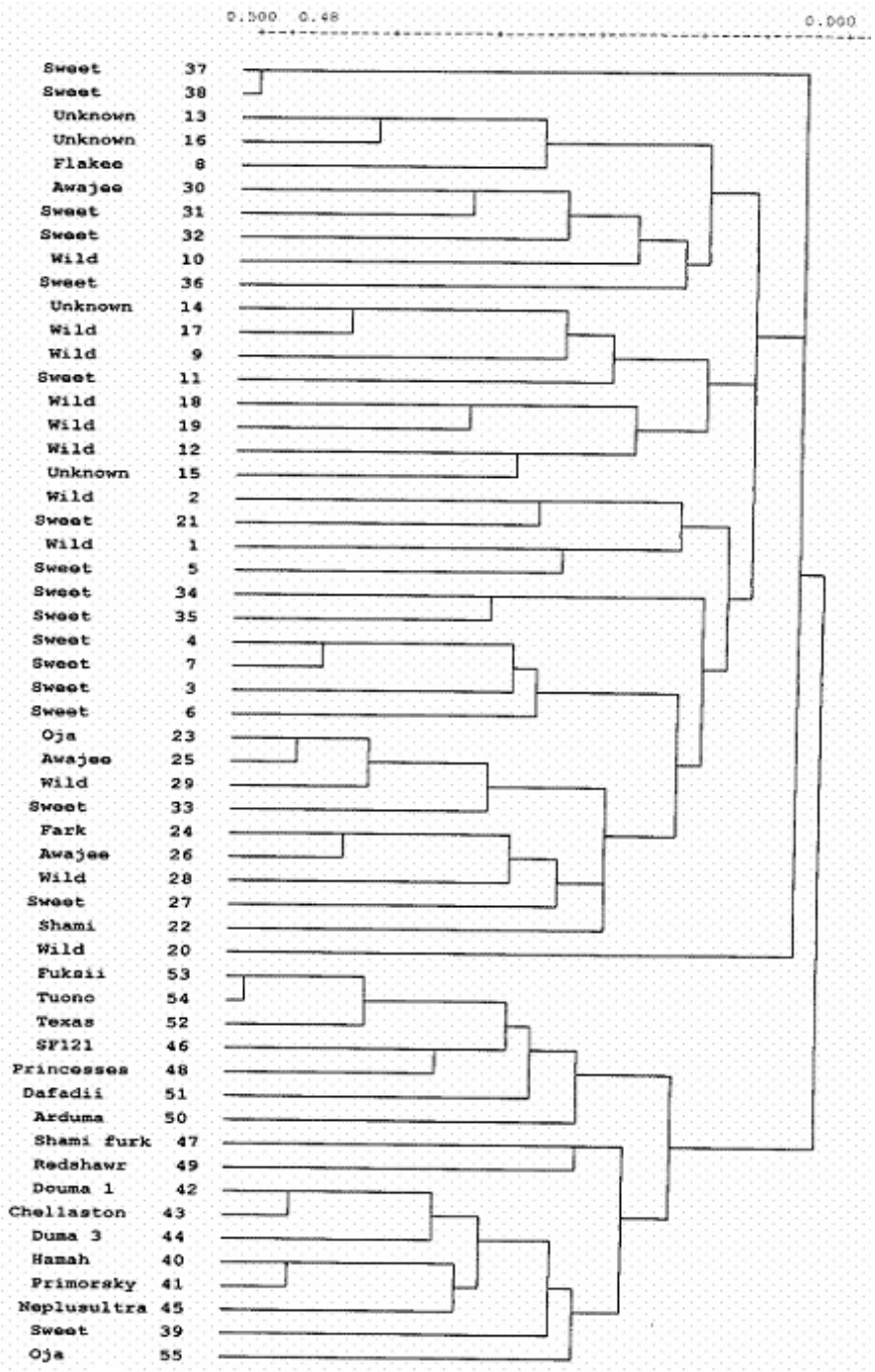

Figure 3. A dendrogram among wild type genotype, farmers orchid genotypes and cultivars of almond by using five RAPD primers.

References

Ahrens S, Venkatachalam M, Mistry A, Lapsley K & Sathe S. 2005. Almond (*Prunus dulcis* L.) protein quality. Plant Foods for Human Nutrition 60, 123-128.

Al-Hmoud A, Khreisat Z & Al-Hewian M. 2006. Cultivars of *Prunus dulcis.* Bulletin. NCARE, Jordan. P.44 (In Arabic).

Aradhya MK, Weeks CF & Simon CJ.2004. Molecular characterization of variability and relationship among seven cultivated and selected wild species of *Prunus* L. using amplified fragment length polymorphism. Scientia Horticulturae 103,131-144.

Baránek M, Raddova´ J & Pidra M. 2006. Comparative analysis of genetic diversity in *Prunus* L. as revealed by RAPD and SSR markers. Scientia Horticulturae 108, 253–259.

Casas AM, Igartua1 E, Balaguer G & Moreno MA.1999. Genetic diversity of *Prunus* rootstocks analyzed by RAPD markers. Euphytica 110, 139–149.

Chessa I, Nieddu G. 2005. Analysis of diversity in the fruit tree genetic resources from a Mediterranean island. Genetic Resources and Crop Evolution 52: 267–276.

Doyle JJ & Doyle JL. 1987. A rapid DNA isolation procedure for small quantities of fresh leaf tissue. Phytochemical Bulletin 19,11–15.

Gouta H, Ksia E, Zoghlami N, Zarrouk M & Mliki A. 2007. Genetic diversity and phylogenetic relationships among Tunisian almond cultivars revealed by RAPD markers. The Journal of Horticultural Science and Biotechnology 83(6), 707-712.

Kester DE, Gradziel TM & Grasselly C.1991. Almonds (*Prunus*). In: Moore, J. N., Ballington, H. J. (eds) Genetic resources of temperate fruit and nut crops. Intl Soc Hort Sci, The Netherlands. pp 701–758.

MirAli N & Nabulsi I. 2003. Genetic diversity of almond (*Prunus dulcis*) using RAPD technique. Scientia Horticulturae 98, 461-471.

Martínez -Gómez, P. Raquel Sánchez Pérez, Federico Dicenta, Werner Howad, Pere Arús & Thomas M2007. Almond. In: Genome Mapping and Molecular Breeding in Plants: Kole (Ed.)Vol. 4, Fruits and Nuts.C.. Springer-Verlag Berlin Heidelberg. P. 230-242

Martins M, Sarmento D & Oliveria MM. 2004. Genetic stability of micro propagated almond plantlets, as assessed by RAPD and IAAR markers. Plant Cell Reproduction 23, 492-496.

Nei, M. and Li, W.H. (1979). Mathematical model for studying genetic variation in terms of restriction endonucleases. Proceedings of the National Academy of Science of the USA 76:5269-5273.

Sarkhosh A, Zamani Z, Fatahi R& Ebadi A. 2006. RAPD markers reveal polymorphism among some Iranian pomegranate (*Punica granatum* L.) genotypes. Scientia Horticulturae 111, 24–29.

Shiran B, Amirbakhtiar N, Kiani S, Mohammadi Sh, Sayed-Tabatabaei BE,& Moradi H. 2007. Molecular characterization and genetic relationship among almond cultivars assessed by RAPD and SSR markers. Scientia Horticulturae 111, 280–292.

Talhouk SN, Lubani RT, Baalbaki R, Zurayk R, AlKhatib A, Parmaksizian L, & Jaradat AA. 2000. Phenotypic diversity and morphological characterization of *Amygdalus* L. species in Lebanon. Genetic Resources and Crop Evolution 12, 93–104.

Vidal JR, Coarer M & Defontaine A. 1999. Genetic relationships among grapevine varieties grown in different French and Spanish regions based on RAPD markers Euphytica 109, 161–172.

Williams JG, Kubelik AR, Livak KJ, Rafalski JA & Tingey SV. 1990. DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucleic Acids Research 18,6531-6535.

Wünsch A & Hormaza JI. 2002. Cultivar identification and genetic fingerprinting of temperate fruit tree species using DNA markers. Euphytica 125, 59–67.

Zeinalabedini M, Majourhat K, Khayam-Nekoui M, Grigorian V, Toorchi M, Dicenta F & Martínez-Gomez P. 2007. Molecular characterization of almond cultivars and related wild species using nuclear and chloroplast DNA markers. Journal of Food, Agriculture & Environment 5, 242-247.

JJBS

Volume 2, Number 2, June 2009
ISSN 1995-6673
Pages 97 - 102

Jordan Journal of Biological Sciences (Short Communication)

Sap11/Sap12 and *egc* Associated Toxin Genes are Dominant in Slime Forming Clinical Staphylococcus Aureus Isolates Harboring *icaABCD-Operon*

Ala'a M. Bawadi, Salwa Bdour and Adel Mahasneh*

Department of Biological Sciences, Faculty of Science, University of Jordan, Amman Jordan

Abstract

The prevalence of adhesion (*icaA* and *icaD*) and toxin (*tst, eta,* and *etb*) genes was studied in 100 clinical *Staphylococcus aureus* isolates. The *icaA, icaD*, *eta*, *etb* and *tst* genes were detected by PCR in 91%, 91%, 2%, 1% and 43% of the isolates, respectively. Various gene combinations with the previously reported enterotoxin genes (*sea*, *sec*, *sei*, *seg* and *seh*) were detected in 47 of 100 isolates. These combinations fall into 3 groups: group I which includes one *eta+sea* containing isolate, group II which includes 25 (53.2%) isolates lacking *tst* gene but harboring *icaA+icaD* genes in 11 combinations with the enterotoxin genes and group III which includes 21 (44.7%) isolates harboring *icaA+icaD+tst* in 7 different combinations with the enterotoxin genes. The predominant gene combinations in group II and III isolates include *seg* + *sei* or *seg* of the enterotoxin gene cluster (*egc*). Furthermore, 20 isolates of group III have the corresponding gene combination profiles of 20 isolates of group II in addition to the *tst* gene. This could be attributed to loss or acquisition of SaPI1 and/or SaPI2 islands which carry the *tst* gene.

The dominancy of SaPI1/SaPI2 and *egc* associated toxin genes in the slimy isolates of *S. aureus* may be understood in the context of pathogenicity functioning genes. This data would contribute to the control of colonization and spread of these isolates in hospitals and community at large.

الملخص

في هذه الدراسة تحديد جينات الالتصاق من فئة icaA, icaD ، وكذلك جينات السمية من فئات efb و eta و tst في 100 عزلة سريرية من البكتيريا العنقودية الذهبية S.aureus وقد تم وجود جينات icaA و icaA و etb و tst بنسب 91، 91، 2، 1، 43% في العزلات على التوالي وذلك باستعمال تقنية تفاعل انزيم البوليمريز المتسلسل (PCR) . كما ولوحظ وجود توليفات من جينات مختلفة مع تلك الموجودة في الأدب العلمي من فئات seg, sei, sec, sea seh في 47 عزلة من المئة التي تمت دراستها. وتقع هذه التوليفات في 3 مجموعات. المجموعة الأولى والتي تشمل عزلة واحدة تحوي الجينات sea + eta والمجموعة الثانية تشمل 25 عزلة تفتقر للجين tst إلا أنها تحمل الجينات icaA + icaD متمثله في 11 توليفة من جينات السمية أما المجموعة الثالثة فتشمل 21 عزلة تحمل جينات tst + icaD + icaA موزعة في سبع توليفات مع جينات السمية. أما التوليفات السائدة في المجموعتين الثانية والثالثة فتشمل sei + seg أو seg من المجموعة الجينية السمية egc.

إضافة لذلك فإن عشرين عزلة من المجموعة الثالثة تحمل أنماط التوليفات المعادلة في عشرين عزلة من المجموعة الثانية إضافة للجين tst. ويمكن أن نغزي ذلك إما الى حمل أو فقد الجزر المرضية من SaPI1 و أو SaPI2 التي تحمل جين tst.

إن سيادة جينات SaPI2 و SaPI2 وجينات السمية المرتبطة بحين ege في عزلات المكورات العنقودية الذهبية الفروية يمكن فهمه في سياق الجينات الامراضية الفاعلة ويمكننا القول بأن نتائج هذه الدراسة سوف تساهم في السيطرة على التموضع وانتشار مثل هذه العزلات في بيئات المستشفيات والمجتمع بصورة عامة.

Keywords: Staphylococcus Aureus; Adhesion Genes; Toxin Genes; Jordan.

1. Introduction

Staphylococcus aureus is an important pathogen causing a variety of diseases in both human and animals (Archer, 1998; Vasudevan *et al*., 2003). Its pathogenesis is attributed to combined effect of toxins and extracellular factors encoded by different genes. These include: superantigens [enterotoxins (SEs), toxic shock syndrome toxin-1 (TSST-1), exfoliative toxins (ETA and ETB)], fibronectin, collagen and fibrinogen binding proteins, in addition to proteins involved in biofilm formation (Baron *et al*., 1994; Palma *et al*., 1996, 1998; Becker *et al*., 1998; Cramton *et al*., 1999; Jarraud *et al*., 2001; Peacock *et al*., 2002; Cucarella *et al*., 2004; Fueyo *et al*., 2005).

Biofilm formation requires production of the extracellular poly-*N*-acetylglucosamine (PNAG) by *icaABCD*-operon encoded enzymes (Cramton *et al*., 1999; Fitzpatrick *et al*., 2005a,b). The *icaA* gene encodes *N*-acetylglucosaminyltransferase which is involved in the synthesis of *N*-acetylglucosamine oligomers from UDP-*N*-acetylglucosamine (Arciola *et al*., 2001a).

* Corresponding author. amahasneh@ju.edu.jo.

The *icaD* gene is involved in expression of *N*-acetylglucosaminyltransferase (Gerke *et al*., 1998). *S. aureus* isolates harboring the *ica* gene cluster are responsible for chronic or persistent infections which can be more problematic due to the presence of antibiotic resistance genes (Stewart and Costerton, 2001; Victoria *et al*., 2002).

Since virulent *S. aureus* strains pose a real problem by increasing healthcare cost worldwide, both in hospitals and in community (Costerton *et al*., 1999), these strains have been identified in several countries in the quest of controlling their spread (Omoe *et al*., 2002; Peacock *et al*., 2002; Becker *et al*., 2003). In previous studies, the prevalence of the staphylococcal enterotoxin genes (*sea-see* and *seg-sej*) was investigated in Jordanian clinical *S. aureus* isolates. Only *sea*, *sec*, *seg*, *sei* and *seh* were detected in 15%, 4%, 37%, 24% and 4% of the total isolates, respectively (Naffa *et al*., 2006; El-Huneidi *et al*., 2006). In this study, we followed the presence of toxic shock syndrome (*tst*), exfoliative toxin (*eta* and *etb*), and the adhesion (*icaA* and *icaD*) genes in the above mentioned isolates. Targeting such additional genes provides further epidemiological information on the toxigenicity of the Jordanian isolates and their ability to form biofilms. This information may also contribute to the control of colonization and the spread of these isolates in the Jordanian hospitals and community.

2. Materials and Methods

2.1. Bacterial Isolates.

A total of 100 clinical *S. aureus* isolates recovered from patients with invasive *S. aureus* diseases admitted to the Jordan University Hospital (Al-Zu'bi, 2004), were included in this study. All isolates were identified by biochemical tests (Daghistani *et al*., 2000).

The following *S. aureus* reference strains were used as positive controls for the studied genes: CECT 975 (*icaA* and *icaD* positive) was kindly provided by the Spanish Type Culture Collection (CECT); *S. aureus* E-1 (*eta* positive) and TY4 (*etb* positive) strains were kindly provided by Dr. Motoyuki Sugai, Graduate School of Biomedical Sciences, Dept. of Bacteriology, Hiroshima University, Hiroshima-Japan; NCTC 11963 (*tst* positive) was purchased from the National Collection of Type Culture (NCTC).

2.2. Slime-Production

The phenotypic ability of *S. aureus* isolates for slime-production was determined by cultivation on Congo red agar (CRA) plates (Freeman *et al*., 1989; Montanaro *et al*., 1999; Arciola *et al*., 2001a; Vasudevan *et al*., 2003; Cucarella *et al*., 2004). CRA plates were incubated for 24 h at 37^{o}C and subsequently overnight at room temperature. Slime producing isolates form black colonies, whereas non-producing isolates develop red colonies. The result was confirmed by amplification of *icaA* and *icaD* genes using the polymerase chain reaction technique (PCR).

2.3. Detection of Adhesion and Toxin Genes

Cell lysate of *S. aureus* (Van de Klundert and Vligenthard, 1993) containing both chromosomal and plasmid DNA was used in 25 µl of PCR reaction mixture. Table 1 shows the sequence and the quantity of primers, amplification conditions and anticipated sizes of PCR products for the tested genes. PCR amplifications were performed in a PE-9600 thermocycler (Perkin-Elmer) using PCR Master Mix (Promega, USA). A positive PCR control containing cell lysate of a reference *S. aureus* and a negative PCR blank with nuclease free water instead of the cell lysate were included with each set of five reactions. After amplification, 10µl of each PCR mixture was analyzed in 1.5% agarose gel, and photographed using the Gel documentation system (UVP, USA).

2.4. Statistical Analysis

The correlation coefficient (r) between the slime producing isolates and the *icaA* and *icaD* gene harboring isolates was calculated using the correlation coefficient (r) formula. Test statistic was used to accept or reject the null hypothesis that there is a significant difference in prevalence of corresponding gene combinations in isolates. *P*-value was calculated at 95% confidence interval using normal distribution tables. $p < 0.05$ was considered statistically significant (Johnson and Bhattacharyya, 1996).

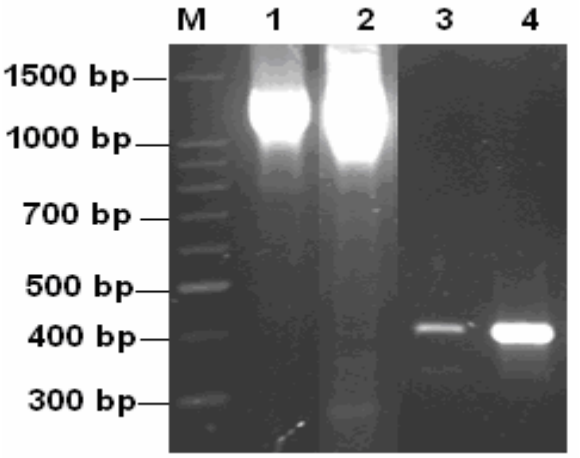

Figure 1. Representative ethidium bromide-stained 1.5% agarose gel analysis of PCR-amplified adhesion genes sequences. M, 100 bp marker (Promega, USA); Lanes 1 and 3, *icaA* and *icaD* positive PCR control (*S. aureus* CETC 975), respectively; Lane 2 and 4, *icaA* and *icaD* positive clinical isolate, respectively.

3. Results

The prevalence of intercellular adhesion and toxin genes is presented in Table 2. Both *icaA* and *icaD* were detected in 91% of the clinical *S. aureus* isolates. The positive isolates produced 1315 bp and 381 bp PCR products, for the *icaA* and *icaD* genes, respectively (Figure 1). The presence of the *icaA* and *icaD* genes was positively associated with the slime production (r = 1). These genes were detected in all isolates that showed black colonies on CRA.

The prevalence of *tst* gene in the clinical isolates was 43%. The *tst* positive isolates produced 180 bp PCR products (Figure 2). Only 2% and 1% of the clinical isolates were positive for the *eta* and *etb* genes, respectively. The *eta* and *etb* positive clinical isolates produced 190 bp and 612 bp PCR products, respectively (Figure 2).

Table 1. Primers, amplification conditions, primer concentration and anticipated sizes of PCR products for tested genes.

Gene	Primer	Oligonucleotide sequence 5'→3'	Size of amplified product (bp)	Primer concentration	Amplification conditions* and No. of cycles	Reference Strain	References
icaA	ICAAF ICAAR	F:5'-CCTAAC TAA CGA AAG GTA G-3' R: 5'-AAG ATATAG CGA TAA GTG C-3'	1315	1 µM	45 sec, 92 C; 45 sec, 49 C; 1 min, 72 C; 35 cycle	CECT975	Vasudevan et al., 2003
icaD	ICADF ICADR	F:5'-AAA CGT AAG AGA GGT GG-3' R: 5'-GGC AAT ATG ATC AAG ATA C-3'	381	1 µM		CECT975	
tst	TST-1 TST-2	F:5'-TTC ACT ATT TGT AAA AGT GTC AGA CCC ACT-3' R: 5'-TAC TAA TGA ATT TTT TTA TCG TAA GCC CTT-3'	180	20 pmol	30 sec, 94 C; 30 sec, 55 C; 1 min, 72 C; 35 cycle	NCTC 11963	Jarraud et al., 2002
eta	mpETA-1 mpETA-3	F:5'-ACT GTA GGA GCT AGT GCA TTT GT-3' R: 5'-TGG ATA CTT TTG TCT ATC TTT TTC ATC AAC-3'	190	20 pmol		E-1	
etb	mpETB-1 mpETB-2	F:5'-CAG ATA AAG AGC TTT ATA CAC ACA TTA C-3' R: 5'-AGT GAA CTT ATC TTT CTA TTG AAA AAC ACT C-3'	612	20 pmol		TY4	

*Initial denaturation at 94°C for 2 min at the beginning of PCR; Final extension at 72° C for 10 min at the end of the cycles.

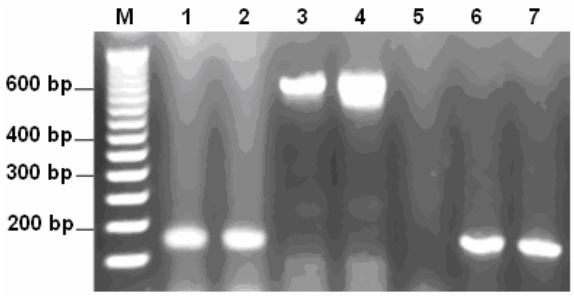

Figure 2. Representative ethidium bromide-stained 1.5% agarose gel analysis of PCR-amplified toxin genes sequences. M, 50 bp marker (Promega, USA); Lane 1, eta positive PCR control (E-1); Lane 2, eta positive clinical isolate; Lane 3, etb positive PCR control (TY4); Lane 4, etb positive clinical isolate; Lane 6, tst positive PCR control (S. aureus NCTC 11963); Lanes 7, tst positive clinical isolates; Lane 5, negative PCR blank.

Table 2. The prevalence and coexistence of adhesion (icaA, icaD) and toxin (tst, eta, etb) genes in 100 clinical S. aureus isolates.

Genes	No. (%) of positive isolates
Single possession of genes:	
icaA	91 (91)
icaD	91 (91)
tst	43 (43)
eta	2 (2)
etb	1 (1)
Multiple possession of genes:	
icaA + *icaD*	91 (91)
icaA + *icaD* + *eta*	1 (1)
icaA + *icaD* + *tst*	42 (42)

The coexistence of the studied adhesion and toxin genes in the *S. aureus* isolates is shown in Table 2. All isolates harboring the *icaA* gene were also harboring *icaD* gene. Only one *eta* containing isolate was positive for *icaA* and *icaD* genes. Most of the *tst* containing isolates (42/43) were positive for *icaA* and *icaD* genes. None of the isolates that contained *eta* or *etb* were positive for *tst* gene and the *etb* harboring isolates did not show any of the *eta*, *icaA* or *icaD* genes (Table 2).

Gene combination with the previously studied enterotoxin genes (*sea*, *sec*, *seg*, *seh* and *sei*) of the same 100 isolates (El-Huneidi *et al*., 2006; Naffa *et al*., 2006) was observed in 47 clinical isolates which fall into three groups (Table 3). These groups differ in the profile of gene combinations. Group I includes one *eta* + *sea* containing isolate which lacks *icaA*, *icaD* and *tst* genes. Group II includes 25 (53.2%) isolates lacking *tst* gene but harboring *icaA* + *icaD* genes in 11 different combinations with the enterotoxin genes. Group III includes 21(44.7%) isolates harboring *icaA* + *icaD* + *tst* in 7 different combinations with the enterotoxin genes. Interestingly, 20 isolates of Group III (gene combination number 1-6) have the corresponding gene combination profiles of 20 isolates of group II (gene combination number 1-6) in addition to the *tst* gene. Only one isolate of Group III (gene combination number 7) does not have corresponding gene combination in Group II isolates. The most frequent (17%) gene combination profile was detected in group II isolates. There was no significant difference ($p > 0.05$) between the prevalence of this combination and the corresponding one (8.5 %) in group III isolates harboring the additional *tst* gene. The second frequent (12.8%) combination profile was detected in group III isolates. There was no significant difference ($p > 0.05$) between the prevalence of this combination and its corresponding one in group II isolates, which represents the third frequent (10.6%) combination. Similarly, there was no significant difference ($p > 0.05$) between the prevalence of other combination profiles in group II isolates and their corresponding ones in group III isolates. A limited number of group II isolates (gene combination number 7-11) do not have corresponding combination profile in Group III isolates. On the other hand, only one isolate in group III harboring *icaA* + *icaD* + *tst* + enterotoxin genes and does not have corresponding combination profile in Group II isolates.

4. Discussion

This study presents an analysis of some virulence determinants in 100 clinical *S. aureus* isolates. It demonstrates the existence of the chromosomal *icaA* and *icaD* genes in 91% of *S. aureus* isolates. The coexistence

of these genes is correlated with slime production on Congo red agar in 91 % of *S. aureus* isolates. The high prevalence of these genes is consistent with that reported by other investigators (Montanaro *et al*., 1999; Ando *et al*., 2004; Peacock *et al*., 2002; Ando *et al*., 2004) and emphasizes the implication of these genes as virulence markers in biofilm formation. Both *icaA* and *icaD* genes are involved in formation of the capsular polysaccharide that also allows the bacteria to escape the immune system (Cramton *et al*., 1999; Arciola *et al*., 2001a, b; Vasudevan *et al*., 2003; Ando *et al*., 2004). However, the failure in slime production by 9% of *S. aureus* isolates might not affect formation of the biofilm, if yet unidentified *ica*-independent mechanisms of biofilm formation or a virulence determinant analogous to Biofilms Associated Protein (BAP) exists in these isolates. BAP is a surface protein and was detected in 5% of bovine mastitis *S. aureus* isolates (Cucarella *et al*., 2004). This protein promotes both primary attachment to inert surfaces and intercellular adhesion and is sufficient to induce biofilm production on abiotic surfaces when the *ica* locus product is absent (Cucarella *et al*., 2004).

In this report, few clinical isolates were harboring the exfoliative toxins genes (*eta* and *etb*). Therefore, screening for these genes in larger samples is necessary to give better information about the prevalence of such genes. The low frequency of these genes is in agreement with the results of other investigators, where Mehrotra *et al*. (2000) found that none of the 107 Canadian nasal isolates were positive for both *eta* and *etb* while the three clinical isolates were positive for both *eta* and *etb*. In Germany, 0.5% of the blood isolates were *eta* positive and none were *etb* positive, while 1.9% and 1% of the nasal isolates were *eta* and *etb* positive, respectively (Becker *et al*., 2003). The limited distribution of these genes suggested that certain isolates acquired the genes by horizontal gene transfer through plasmids or temperate bacteriophages. The *etb* gene is located on large plasmids while *eta* gene is carried on the genome of a temperate phage integrated in the *S. aureus* genome (Yamaguchi *et al*., 2001).

Results of this study showed a high prevalence (43%) of *tst* in the clinical isolates (Table 2) which could be correlated with the transfer of this gene at high frequency (Moore and Lindsay, 2001). Similar prevalence (40%) of *tst* was reported in methicillin-susceptible *S. aureus* (MSSA) at the University Hospital in Magdeburg, Germany (Layer *et al*., 2006). Higher prevalence (72.5%) was detected in Japanese clinical isolates (Ando *et al*., 2004). Lower prevalence was detected in German blood (18.3%) and nasal isolates (22.4%) (Becker *et al*., 2003), Polish nasal isolates (10.5%) (Bania *et al*., 2006), German animal isolates (15.5%) (Akineden *et al*., 2001), and other animal isolates (26.7%) from different countries (Smyth *et a*l., 2005). Most (42/43) Jordanian *tst* positive isolates are slime formers (Table 2) and 50% (21/42) of these isolates contain various combinations of enterotoxin genes (Table 3). The profile of gene combination (number 1-6) in 20 *tst* positive isolates (group III, Table 3) is analogous to that detected in other 20 isolates of group II lacking *tst* gene The presence and absence of *tst* in these groups of isolates can be attributed to the presence of *tst* on a mobile genetic element called staphylococcal pathogenicity islands (SaPI1 and SaPI2) (Schmidt and Hensel, 2004). Loss or acquisition of these islands could be the mechanism that contributes to the appearance of these groups of isolates. Transduction of SaPI1 and SaPI2 by helper phages was demonstrated (Lindsay *et al*., 1998; Ruzin *et al*., 2001). In the absence of these helper phages, these islands remain stably integrated in the chromosome (Schmidt and Hensel, 2004). A pathogenicity island (SaPIbov) related to SaPI1 was identified in bovine isolates of *S. aureus* (Fitzgerald *et al*., 2001). SaPIbov harbors *tst*, *sec* and *sel* genes (Schmidt and Hensel, 2004). The co-existence of the *tst* and *sec* genes in animal isolates including bovine has been reported (Akineden *et al*., 2001; Fitzgerald *et al*., 2001; Smyth *et al*., 2005). This contrasts with findings of rare co-existence of *tst* and *sec* genes in 3 clinical isolates of Group III in the present study and in other studies (Peacock *et al*., 2002; Becker *et al*., 2003; Bania *et al*., 2006; Layer *et al*., 2006) and confirms that SaPI1 and SaPI2 lack *sec*.

Several investigators (Peacock *et al*., 2002; Becker *et al*., 2003; Layer *et al*., 2006) suggested that a number of bacterial determinants act in combination during the infective process. The presence of various gene combinations in 46 slime forming isolates in this study (groups II and III, Table 3) supports some sort of association between pathogenicity and colonization genes of *S. aureus*. The predominant gene combinations in these isolates include *seg* + *sei* or *seg*. These genes belong to the enterotoxin gene cluster (*egc*) that was identified by Jarraud *et al* (2001). This finding is consistent with that reported in other countries for human (Becker *et al*., 2003; Bania *et al*., 2006; Layer *et al*., 2006) and animal strains of *S. aureus* (Akineden *et al*., 2001; Smyth *et al*., 2005). The dominancy of the *egc* cluster genes in human and animal isolates suggests a potential role of these superantigens in different infections caused by *S. aureus*.

It is important to mention that PCR is able to demonstrate the existence of genes in *S. aureus* isolates but it does not prove the production of the proteins encoded by these genes. Therefore, bioassay or immunological methods must be used to demonstrate the ability of the Jordanian isolates to produce the toxin and the adhesion proteins.

In conclusion, this study has demonstrated the variable presence of *icaA* and *icaD*, *tst, eta,* and *etb* genes in the clinical isolates of *S. aureus*. These genes also coexist in different combinations with the previously detected enterotoxin genes (Naffa *et al*., 2006; El-Huneidi *et al*., 2006), supporting the notion that these genes act in combination during infection. This data may help in providing a guideline for the control of colonization and the spread of these isolates in the hospital environment and community.

Acknowledgment

Our thanks are due to Dr. Motoyuki Sugai (Graduate School of Biomedical Sciences, Dept. of Bacteriology, Hiroshima University, Hiroshima, Japan) for providing us with the E-1 (*eta* positive) and TY4 (*etb* positive) *S. aureus* strains.

References

Akineden O, Annemuller C, Hassan AA, Lammler C, Wolter W, Zschock M (2001). Toxin genes and other characteristics of Staphylococcus aureus isolates from milk of cows with mastitis. Clin Diagn Lab Immunol 8: 959-64.

Al-Zu'bi E, Bdour S, Shehabi A (2004). Antibiotic resistance patterns of mecA-positive *Staphylococcus aureus* isolates from clinical specimens and nasal carriage. Microb Drug Resist 10: 321-324.

Ando E, Monden K, Mitsuhata R, Kariyama R, Kumon H (2004). Biofilm formation among methicillin-resistant Staphylococcus aureus isolates from patients with urinary tract infection. Acta Med Okayama 58: 207-214.

Archer G (1998) *Staphylococcus aureus*: A well-armed pathogen. Clin Infect Dis 26: 1179-1181.

Arciola CR, Baldassarri L, Montanaro L (2001a). Presence of *icaA* and *icaD* genes and slime production in a collection of Staphylococcal strains from catheter-associated infections. J Clin Microbiol 39: 2151-2156.

Arciola C, Collamati S, Donati E, Montanaro L (2001b). A rapid PCR method for the detection of slime producing strains of *Staphylococcus epidermidis* and *S. aureus* in periprosthesis infections. Diagnostic Molecular Pathology 10: 130–137.

Bania J, Dabrowska A, Korzekwa K, Zarczynska A, Bystron J, Chrzanowska J, Molenda J (2006) The profiles of enterotoxin genes in *Staphylococcus aureus* from nasal carriers. Lett Appl Microbiol 42: 315-320.

Baron E, Peterson L, Fingegold S (1994). *Diagnostic Microbiology* (9th ed.). United State of America: Mosby.

Becker K, Roth R, Peters G (1998). Rapid and specific detection of toxigenic *Staphylococcus aureus*: use of two multiplex PCR enzyme immunoassays for amplification and hybridization of Staphylococcal enterotoxin genes, exfoliative toxin genes, and toxic shock syndrome toxin-1 gene. J Clin Microbiol 36: 2548-2553.

Becker K, Friedrich A W, Lubritz G, Weilert M, Peters G, von Eiff C (2003). Prevalence of genes encoding pyrogenic toxin superantigens and exfoliative toxins among strains of *Staphylococcus aureus* isolated from blood and nasal isolates. J Clin Microbiol 41: 1434-1439.

Costerton JW, Stewart PS, Greenberg EP (1999). Bacterial biofilm: a common cause of persistent infections. Science 284: 1318-1322.

Cramton S E, Gerke C, Schnell N, Nichols W W, Gotz F (1999). The intercellular adhesion *(ica*) locus is present in *Staphylococcus aureus* and is required for biofilm formation. Infect immun 67: 5427–5433.

Cucarella C, Tormo M, Ubeda C, Trotonda M, Monzon M, Peris C, Amorena B, Lasa I, Penades J (2004). Role of biofilm-associated protein bap in the pathogenesis of bovine Staphylococcus aureus. Infect Immun 72: 2177-2185.

Daghistani H, Issa A, Shehabi A (2000). Frequency of nasal and wound isolates of *Staphylococcus aureus* associated with TSST-1 production in Jordanian population. FEMS Immun Med Microbiol 27: 95-98.

El-Huneidi W, Bdour S, Mahasneh A (2006). Detection of enterotoxin genes seg, *seh*, *sei*, and *sej* and of a novel *aroA* genotype in Jordanian clinical isolates of *Staphylococcus aureus*. Diagn Microbiol Infect Dis. 56: 127-132

Fitzgerald, J R., Monday S R, Foster T J, Bohach G A, Hartigan P J, Meaney W J, Smyth C J (2001). Characterization of a putative pathogenicity island from bovine *Staphylococcus aureus* encoding multiple superantigens. J Bacteriol 183:63-70.

Fitzpatrick F, Humphreys H, Gara JPO. (2005a). The genetics of staphylococcal biofilm formation- will a greater understanding of pathogenesis lead to better management of device-related infection?. Clin Microbiol Infect 11: 976-973.

Fitzpatrick F, Humphreys H, Gara JPO. (2005b). Evidence for *icaABCD*-independent biofilm development mechanism in methicillin-resistant *Staphylococcus aureus* clinical isolates. J Clin Microbiol 43 : 1973-1976.

Fueyo JM, Mendoza MC, Rodicio MR, Muniz J, Alvarez MA, Martin MC (2005) Cytotoxin and pyrogenic toxin superantigen gene profiles of *Staphylococcus aureus* associated with subclinical mastitis in dairy cows and relationships with macrorestriction genomic profiles. J Clin Microbiol 43: 1278-1284.

Gerke C, Kraft A, Sussmuth R, Schweitzer O, Gotz F (1998). Characterization of the *N*-acetylglucosaminyltransferase activity involved in biosynthesis of *Staphylococcus epodermidis* polysaccharide intercellular adhesion. J Biol Chem 27: 18586-18593.

Jarraud S, Peyrat M, Lim A, Tristan A, Bes M, Mougel C, Etienne J, Vandenesch F, Bonneville M, Lina G (2001). *egc,* a highly prevalent operon of enterotoxin gene, forms a putative nursery of superantigens in *Staphylococcus aureus*. J Immunol 166: 669-677.

Jarraud S, Mougel C, Thioulouse J, Lina G, Meugnier H, Forey F, Nesme X, Etienne J, Vandenesch F (2002). Relationships between *Staphylococcus aureus* genetic background, virulence factors, *agr* groups (alleles), and human disease. Infect Immun 70, 631-641.

Johnson R A, Bhattacharyya G K (1996). Biostatistics, Principles and Methods (3rd ed.). New York: John Wiley and Sons; 99-108; 380-448.

Layer F, Ghebremedhin B, Konig W, Konig B (2006). Heterogeneity of methicillin-susceptible *Staphylococcus aureus* strains at a German university hospital implicates the circulating-strain pool as a potential source of emerging methicillin-resistant *S. aureus* clones. J clin Microbiol 44: 2179-2185.

Lindsay J A, Ruzin A, Ross H F, Kurepina N, Novick R P (1998). The gene for toxic shock *toxin is carried by a family of mobile pathogenicity islands in Staphylococcus aureus. Mol Microbiol* 29:527-543

Mehrotra M, Wang G, Johnson W M (2000). Multiplex PCR for detection of genes for *Staphylococcus aureus* enterotoxins, exfoliative toxins, toxic shock syndrome toxin-1, and methicillin resistance. J Clin Microbiol 38: 1032-1035.

Montanaro, L., Arciola, C. R., Baldassarri, L. and Borsetti, E. (1999). Presence and expression of collagen adhesin gene (*cna*) and slime production in *Staphylococcus aureus* strains from orthopaedic prosthesis infections. Biomaterials 20: 1945-1949.

Moore P C L, Lindsay J A (2001). Genetic variation among hospital isolates of methicillin-Sensitive *Staphylococcus aureus*: evidence of horizontal transfer of virulence genes. J Clin Microbiol 39: 2760-2767.

Naffa RG, Bdour SM, Migdadi HM, Shehabi AA (2006). Enterotoxicity and genetic variation among clinical *Staphylococcus aureus* isolates in Jordan. J Med Microbiol 55: 183-7.

Omoe K, Ishikawa M, Shimoda Y, Hudlueda S, Shinagawa K (2002). Detection of *seg, seh*, and *sei* genes in *Staphylococcus aureus* isolates and determination of the enterotoxin productivities of *S. aureus* isolates harboring *seg, seh*, or *sei* gene. J Clin Microbiol 40: 857-62.

Palma, M., Nozohoor, S., Schennings, T., Heimdahl, A. and Flock, J. (1996). Lack of the extracellular 19-kilodalton

fibrinogen-binding protein from *Staphylococcus aureus* decreases virulence in experimental wound infection. Infect Immun 64: 5284-5289.

Palma, M., Wade, D., Flock, M. and Flock J. (1998). Multiple binding sites in the interaction between an extracellular fibrinogen-binding proein from *Staphylococcus aureus* and fibrinogen. J Biol Chem 273: 13177-13181.

Peacock, S. J., Moore, C. E., Justice, A., Kantzanou, M., Story, L., Mackie, K., O'Neill, G. and Day, N. P. J. (2002). Virulent combinations of adhesin and toxin genes in natural populations of *Staphylococcus aureus*. Infect Immun 70: 4987-4996.

Ruzin A, Lindsay J, Novick R P (2001). Molecular genetics of SaPI1-a mobile pathogenicity island in *Staphylococcus aureus.* Mo. Microbiol 41:365-377

Schmidt H, Hensel M (2004). Pathogenicity Islands in Bacterial Pathogenesis**.** Clin Microbiol Rev 17: 14-56**.**

Smyth DS, Hartigan PJ, Meaney WJ, Fitzgerald JR, Deobald CF, Bohach GA, Smyth CJ (2005). Superantigen genes encoded by the *egc* cluster and SaPIbov are predominant among *Staphylococcus aureus* isolates from cows, goats, sheep, rabbits and poultry. J Med Microbiol 54: 401-411.

Stewart P S, Costerton JW (2001). Antibiotic resistance of bacteria in biofilms.. Lancet 358: 135-138.

Van de Klunder, J. and Vliegenthart, J. (1993). Diagnostic Molecular Microbiology, Principle and Applications (2nd ed.). USA: American Society for Microbiology.

Vasudevan P, Nair M, Annamalai T Venkitanarayanan K (2003). Phenotypic and genotypic characterization of bovine mastitis isolates of *Staphylococcus aureus* for biofilm formation. Vet Microbiol 92: 179-185.

Victoria J, Lopez M, Perez-Roth E, Claveria-Martin F, Gil O D, Batista N, Morales M, Mendez-Alvarez S. (2002). Detection of *Staphylococcus aureus* clinical isolates harboring the *ica* gene cluster needed for biofilm establishment. J Clin Microbiol 40: 1569-1570.

Yamaguchi T, Hayashi T, Takami H, Nakasone K, Ohnishi M, Nakayama K, Yamada S, Komatsuzawa H, Suagi M. (2000). Phage conversion of exfoliative toxin A production in *Staphylococcus aureus*. Mol Microbiol 38: 694-705.

Yamaguchi T, Hayashi T, Takami H, Ohnishi M, Murata T, Nakayama K, Asakawa K, Ohara M, Komatsuzawa H, Suagi M. 2001. Complete nucleotide sequence of *Staphylococcus aureus* exfoliative toxin b plasmid and identification of a novel ADP-ribosyltransferase, EDIN-C. Infect Immun 69: 7760-7771.

المملكة الأردنية الهاشمية

الجامعة الهاشمية

المجلة الأردنية
للعلوم الحياتية

JJBS

مجلة علمية عالمية محكمة

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

ISSN 1995-6673

المجلة الأردنية للعلوم الحياتية
مجلة علمية عالمية محكمة

المجلة الأردنية للعلوم الحياتية : مجلة علمية عالمية محكمة أسستها اللجنة العليا للبحث العلمي في وزارة التعليم العالي والبحث العلمي، الأردن، وتصدر عن عمادة البحث العلمي والدراسات العليا، الجامعة الهاشمية، الزرقاء، الأردن.

ترسل البحوث إلى العنوان التالي :

رئيس تحرير المجلة الأردنية للعلوم الحياتية
عمادة البحث العلمي و الدراسات العليا
الجامعة الهاشمية
الزرقاء – الأردن
هاتف : ٣٩٠٣٣٣٣ ٥ ٠٠٩٦٢ فرعي ٤١٤٧
Email: jjbs@hu.edu.jo
Website: www.jjbs.hu.edu.jo