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



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

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

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## EDITORIAL PREFACE

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

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

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

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

March, 2017

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

JJBS

*Volume* 10, *Number 1, March .2017*
*ISSN 1995-6673*

**Jordan Journal of Biological Sciences**

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## Original Articles

JJBS
Jordan Journal of Biological Sciences

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ISSN 1995-6673
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# A New Record of *Potentilla lignosa* Willd. (Rosaceae) in Iraq-Short Communication

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*Received: December 17, 2016 Revised: January 23, 2017 Accepted: January 29, 2017*

**Abstract**

*Potentilla lignosa* Willd is a new additional species to the Rosaceae family in Iraq, from Qandil mountain (north-east of Erbil) within Rowanduz district (MRO). The identity of the species was confirmed by identification using keys in the available references, morphological description was prepared, and some discriminative characters are given in the associated figures. In addition, some characters of the pollen grains have been studied such as shapes, colors, sizes and numbers.

**Key words:** *Potentilla lignosa*, Rosaceae, Rowandus district, Iraq.

## 1. Introduction

One of the families in the Flora of Iraq is Rosaceae that involves 2800 s pecies throughout the world which, distributed on 95 genera (Simpson, 2006), in Iraq, involves 53 species distributed on 1 9 genera (Al-Rawi, 1964). In the Flora of U.S.S.R., Komarov (1941) indicated 148 species of the genus *Potentilla* L. Matthews (1972) in Turkey mentioned 53 species of the genus involving *Potentilla lignose*. In Europe, Ball et al. (1968) stated 75 species of the genus *Potentilla*. In Iran, Schiman (1969) mentioned that 51 species of the genus found including *Potentilla lignosa*. In the Flora of low land Iraq, Rechinger (1964) stated 1 species. Al-Rawi (1964), Meikle (1966) and Ridda & Daood (1982) indicated that 6 species found in Iraq. Faris (1983) mentioned 1 species in Piramagrun Mountain. None of Khalaf (1980), Fatah (2003) and Ahmad (2013) mentioned any species of the genus *Potentilla* in Sinjar, Haibat Sultan and Hawraman mountains, respectively.

The present study assured the occurrence of *P. lignosa* in Iraq based on recent collection, as well as morphological characters and pollen grains characters, to added extra information to support the identity of this species.

## 2. Materials and Methods

Plant specimens have been collected during the field trips in different regions of northern districts of Iraq in 2016, identification of the specimens were done using some of the keys, especially in Flora of Iraq, Flora of Turkey and Flora Iranica. The specimens were treated and preserved, and placed in herbarium of Education College (ESUH). Species' geographical distribution was presented, association of some ecological notes as shown in the map (Figure 1). For the study of pollen grains, anthers were fixed in FAA; then a single anther was removed and placed in a d rop of water or 50% glycerol (the latter to prevent the material from drying out). The anther was dissected with a s calpel to extrude the pollen grains. The anther wall material was removed after crushing pollen grains. And a drop of safranin was added and then a cover-slip was slided on top of the pollen (Simpson, 2006).

## 3. Results

*Potentilla lignosa* Willd., in Ges. Nat. Freunde Berlin Mag. 7:293 (1816); Fl. Iranica, Schiman, No.42/15.3: 85 (1969); Fl. Turkey, Matthews, 4: 45 (1972). Syn: *P. plagiophylla* Rech. fil. in Symb. Bot. Upsal. 11(5):24, t. 12 (1952).

Dwarf suffruiticose with thick woody branches adpressed to rocks, pilose-pubescent, Perennial, herbs, 6-15 cm, stem erect-ascending, pilose-pubescent, green, 2.5-9x0.2-1 cm. Leaves alternate, Leaves compound, petiolate, leaflets 5, terminal ones the largest, oblanceolate, margin entire, apex obtuse, 3-5 toothed, base acute or oblique, pilose-pubescent, green, basal leaves 14-16x7-9 mm, leaflets 5.5-8x2.5-4.5 mm, lower cauline leaves 19-24x9-11 mm, leaflets 5.2-7x2-3 mm, upper cauline leaves 10-12.5x7-9 mm, leaflets 4-6.2x2-4 mm, stipules adnate, auriculate, brown, pubescent, 1.5-3.5x1-1.3 mm. Bracts 2, opposite, narrowly oblong, lanceolate-narrowly elliptic, margin entire, apex acutuminate, base obtuse, pubescent, brown, 1.5-2x0.4-0.6 mm. Flowers terminal, solitary or paired, 10-12x14-17 mm, pedicel teret, pilose-pubescent, green-yellow or green-brown, 15-25x2-3 mm, epicalyx segments 5, linear, pilose-pubescent, green, 1.7-2.5x0.4-0.6 mm. Calyx of 5 sepals, persistent, lanceolate or oblong, margin entire, apex acuminate or acute-acuminate, base truncate, pilose-pubescent, green, 4-6x1.5-2.6 mm. Corolla of 5 pe tals, suborbicular-orbicular, margin undulate, apex obtuse and emarginate, base truncate, unguiculate

glabrous, white, 4.7-6.6x2.8-3.7 mm, Stamens 17-20, filaments filiform, pink, 2-3x0.15-0.20 mm, anthers oblong, pink, versatile attechment with the filaments, 0.8-1x0.25-0.30 mm. Pollens yellow, single, tr icolporate, oblate-prolate in equatorial view, triangular-spheroid in polar view, small according to (Erdtman, 1971), equatorial axis 12-14 µm, polar axis 10-13 µm, numerous. Pistils 8-10, ovaries superior, oblanceolate-oblong, pilose-pubescent, brown, 0.8-1.7x0.4-0.5 mm, style sub-basal, filiform, pink, 3.5-4.6x0.1-0.12 mm. Stigma undifferentiated. Persistent epicalyx 1.8-2.7x0.4-0.7 mm, Persistent sepals 4.8-6.5x2-2.8 mm. Achenes oblanceolate-oblong, sub-basal stylar scar appear on the achenes, pilose-pubescent, brown, 1.1-2x0.5-0.8 mm. Seed single, basal, oblong, yellow, 1-1.6x0.35-0.45 mm. (Plates 1-4).

**Type**: [Iran] Samarische Schneegebirge, Pallas.

**Selected samples from the studied specimens**

**MRO**: ESUH/ Qandil mountain (north-east of Erbil), 2140-2200 m, 25.8.2016, A. Sardar, S. Al-Dabagh and K. Rasul 7451.

**Environment & Geographical Distribution**

Found as individuals in the region, in wet places on the rocks; altitude: 2140-2200 m; flowering: June-August. Found in Qandil mountain within Rowanduz district (MRO) (Figure 1).

**Plate 1**: Field photograph of *P. lignosa*

A part of plant

A part of plant showing branching

Basal leaf: abaxial

Lower cauline leaves: abaxial & adaxial

Upper cauline leaf: adaxial

Upper cauline leaf: abaxial


Adnate stipules

**Plate 2.** Vegetative parts of *P. lignosa*

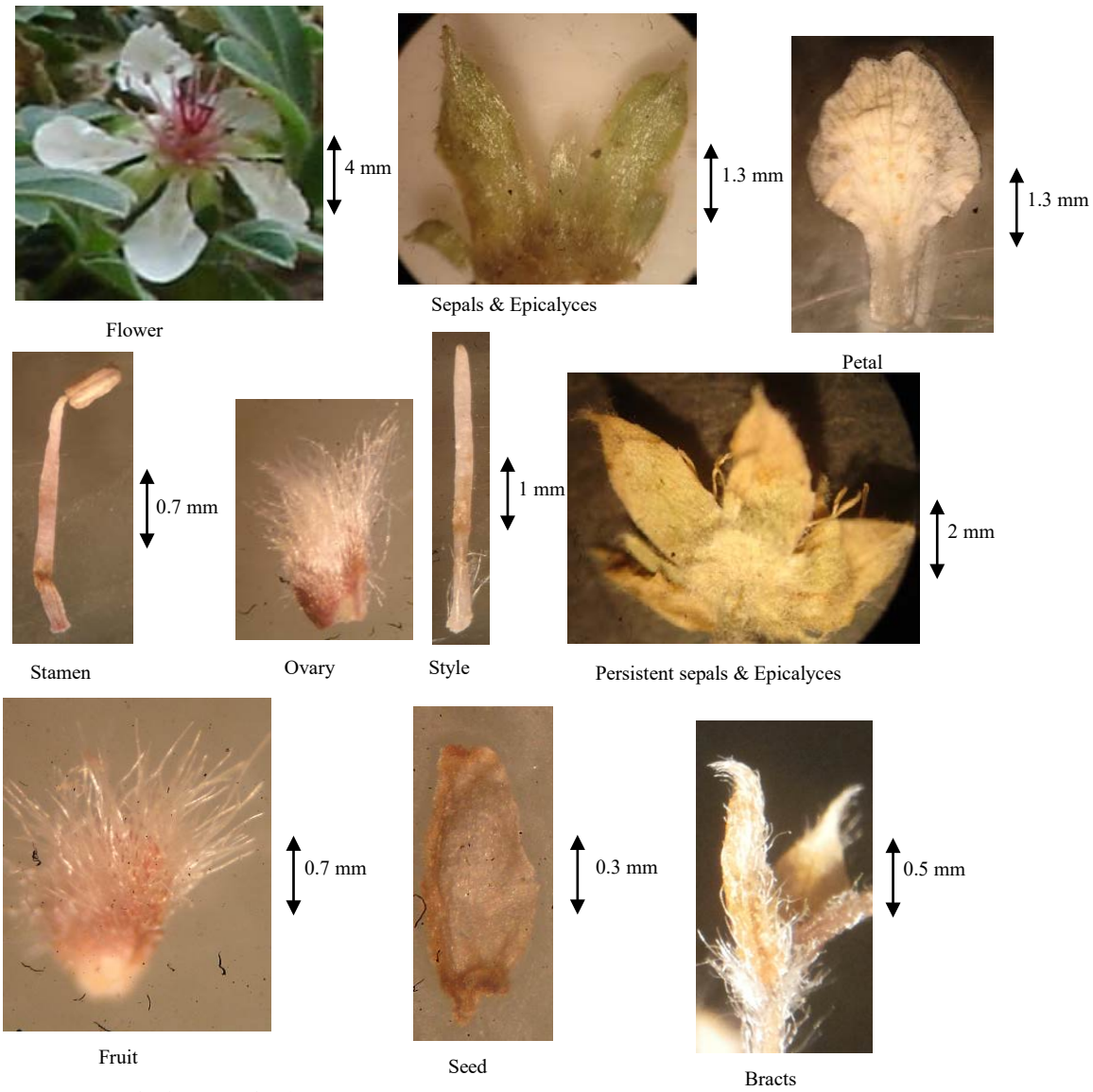


**Plate 3**. Reproductive parts of *P. lignosa*

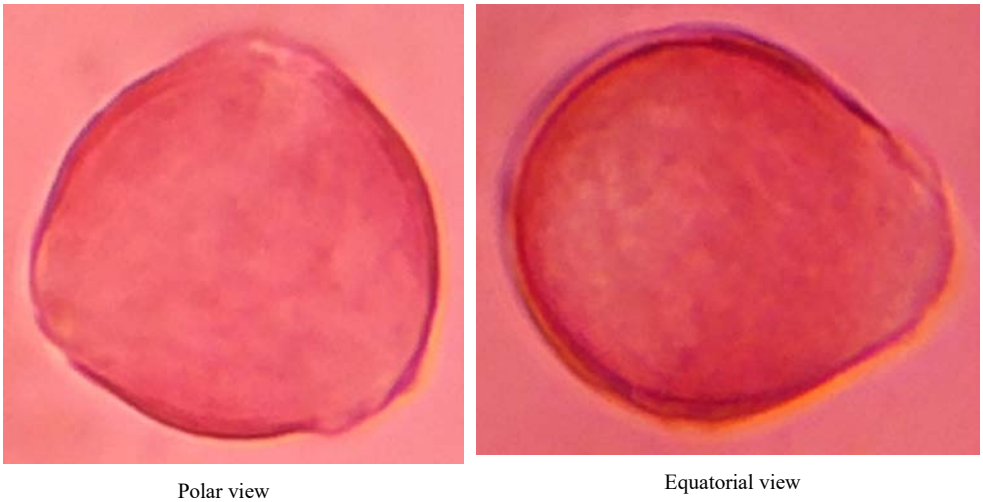


**Plate 4.** pollen grain of *P. lignosa* x100


**Figure 1.** A map of Iraq shows the regions and districts depending on Guest (1966) and FAO (2002) ● *P. lignosa*

## 4. Discussion

The present study dealt with a new record of *Potentilla* species which is *P. lignosa* from Rosaceae family in Iraq, the study included some aspects as the morphological characters and the environment. Within literature review connected to the genus *Potentilla* in Iraq, involving the specimens of National Herbarium of Iraq (BAG), College of Science Herbarium, University of Salahaddin - Erbil, Iraq (ARB) and College of Education Herbarium, University of Salahaddin - Erbil, Iraq (ESUH), the researcher did not find any specimens belongs to *P. lignosa*, therefore it will be regarded as a new species to the Flora of Iraq (new record in Iraq) from Qandil mountain.

*P. lignosa* has some characters different from *P. supina* L. found in Iraq and has pinnately compound leaves (the other Iraqi species have palmately compound leaves), and these characters include that *P. lignosa* is a perennial, dwarf suffruiticose with thick woody branches adpressed to rocks, leaves short petiolate, leaflets 5, 3-5 toothed at apex, stipules auriculate, Flowers terminal, solitary or paired, sepals lanceolate or oblong, acuminate, petals unguiculate, white, Achenes oblanceolate-oblong, pilose-pubescent. In addition, pollen grains were yellow, single, tricolporate, oblate-prolate in equatorial view, triangular-spheroid in polar view, small and numerous.

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JJBS
Jordan Journal of Biological Sciences

Volume 10, Number 1,March 2017
ISSN 1995-6673
Pages 7 - 12

# Effect of Temperature and pH on Egg Viability and Pupation of *Anopheles arabiensis* Patton (Diptera: Culicidae): Prospect for Optimizing Colony Reproduction Procedures

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*Received: November 14, 2016 Revised: December 25, 2016 Accepted: January 7, 2017*

**Abstract**

Optimizing procedures for mass rearing mosquitoes for practices akin to the sterile insect techniques or routine laboratory activities is crucial. The present study evaluated the impact of nutrients, temperature, egg storage period and pH on egg hatchability and pupation rate, respectively, of *Anopheles arabiensis* mosquitoes. First, twenty eggs, collected from female mosquitoes and raised on different diet types (*Rastrineobola argentea*, Tetramin® Baby fish food and Bakers' active yeast) in their larval stages, were stored at different time periods at two temperature regimes; 22 – 23$^{O}$C and 28 – 29$^{O}$C and later dispensed in plastic cups (4.0 cm top × 3.5 cm bottom × 2.7 cm height) containing 25cm$^3$ of water and left to hatch. Secondly, twenty L4s were placed individually in 100 Ml of larval rearing media of different pH regimes in plastic cups (7.5 cm top × 5.0 cm bottom × 8 cm height) and left to pupate and emerge as adults. The media were of pH 6, 6.8 (clean borehole water), 7, 8, 9 and cow dung solution. It was found that eggs incubated at 28-29$^{O}$C were less viable than those incubated at 22-23$^{O}$C ($p < 0.05$). Eggs remained hatchable for 8 days. Mean pupation time for L4 larvae maintained in untreated tap water (pH 6.8) differed significantly compared to other rearing media ($p p < 0.05$). Mean pupation time was neither influenced by sex ($p = 0.124$) or size ($p = 0.801$) of emerged mosquitoes. It was concluded that pH (6.8) and temperatures of 22-23$^{O}$C were optimal for pupation and egg hatchability, respectively.

**Keywords**: Temperature, pH, Storage period, Eggs hatchability, *An. Arabiensis*, Pupation.

## 1. Introduction

Ninety percent of mosquito's life history is aquatic. Eggs, larvae and pupae, referred to as immature stages, are aquatic and thrive differently in different habitat types (Munga *et al*., 2006; Ndenga *et al*., 2011). This is because habitats differ in physical, chemical and biological characteristics (Edillo *et al*., 2006). Emerged mosquitoes or adults are terrestrial. Typically less than 10% of laid eggs emerge as adult (Munga *et al*., 2007; Mwangangi *et al*., 2006; Okogun, 2005).

To optimize on survival, it has been observed that *Anopheles arabiensis*, *Anopheles funestus* (Lyons *et al*., 2013) and *Anopheles gambiae* (Bayoh and Lindsay 2003; Kirby and Lindsay, 2009; Bayoh and Lindsay, 2004; Rocca *et al*., 2009) mosquitoes choose to breed in open, sunlit pools. In such habitats it is believed, temperatures (Small *et al*. 2003; Hoffmann 2010; Parham *et al*., 2012), oxygen (Okogun, 2005), nutrient and pH (Russel, 1999; Tiimub *et al*., 2012) are optimal (Piyaratne *et al*., 2005) for the development of the immatures.

The replication of optimal condition for the generation of mosquito immatures is crucial for mass generation of malaria vectors for studies that require large numbers of mosquitoes for use in the laboratory or for procedures akin to the Sterile Insect Techniques (SIT) that would require millions of mosquitoes (Robinson *et al*., 2009) for purposes of irradiation and subsequent release to inundate and manage nuisance insect population (Knipling, 1955).

A challenge for mass production of anopheline mosquitoes is the fact that freshly laid anopheline eggs are sticky and therefore handling and counting them is tedious and impractical. Moreover, the eggs remain viable for a short time only when kept on wet substrates (Clements, 1992). This means efforts must be directed towards determining the drying and storing conditions that would make it easy to manipulate and count the eggs without compromising viability.

Like all poikilotherms, mosquitoes' biochemical and physiological processes depend on ambient environmental temperature (Couret and Benedict, 2014). Temperature influences the duration and rate of development (Dixon *et al*., 2009), timing of maturation (Yoshioka *et al*., 2012) and body size (Evans *et al*., 2012). It also dictates on humidity (Focks *et al*., 1993) that determines whether the adult mosquitoes aestivate or migrate to other areas of favourable temperatures (Lehmann *et al*., 2010; Adamou *et al*., 2011).

Humidity and temperature influence desiccation and therefore the rate of mosquito egg survival (Juliano *et al*., 2002). It follows that desiccation-resistant eggs will not

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only increase the potential of mosquito colony establishment in non-native habitats but also provide extended shelf life for mosquito eggs meant for procedures akin to the sterile insect techniques

Additionally, anopheline mosquitoes are known filter feeders and under suitable temperatures, pH and oxygen diffusion, acquire optimal nutrients from their aquatic habitats (Sanford, 2005). This, however, is dictated by the amount of Dissolved Oxygen (DO) within the aquatic habitat in such a manner that the higher the DO, the more unsuitable the habitat is to the larvae. Additionally, pH has been observed to alter dissolved oxygen content (Gilvear and Bradley, 2000) by increasing the amount of free ammonia. It follows that pH of larval habitat must be within optimal range because if this is not the case mosquito development is reduced (Curtis, 1996).

Based on this information, it was hypothesized that nutrient, temperature, egg storage period and pH, under which the mosquito parental stocks were exposed, had no effect on egg hatchability and pupation rates of *An. arabiensis* respectively. This study was conducted to:

(1) Evaluate the temperature regime that leads to minimal desiccation of *An. arabiensis* eggs;

(2) evaluate the pH that offers optimal pupation and emergence of *An. arabiensis* adults; and

(3) evaluate the effect of nutrients on *An. arabiensis* size.

## 2. Materials and Methods

### *2.1. Mosquito Colony Origin*

The present study was carried out at the laboratories and insectaries of the School of Biological Sciences, University of Nairobi, Kenya. The *Anopheles arabiensis* mosquito strain used in the present study was originally from Dongola in northern Sudan but was sourced from the International Atomic Energy Agency (Seibersdorf laboratories) in Vienna, Austria. The mosquitoes were cultured at a temperature of 28 – 30 $^{O}$C, relative humidity of 70 - 80% and photoperiod of 12:12 (L:D).

### *2.2. Larval Diet Types*

Three larval diet types were used to rear *An. arabiensis* mosquitoes for the present study. *Rastrineobola argentea* also known as sardine in English was bought from a local market, oven dried, crushed into powder using a food blender and put in a glass vial. Tetramin® Baby fish food was obtained from Seibersdorf laboratories in The Netherlands. Bakers' active yeast was bought from the local supermarket. All the food types were refrigerated at 4$^{O}$C.

### *2.3. Mosquito Stock Culture*

The mosquitoes used in the studies were from the F5 generation onwards and were reared following standard procedure (Dominic *et al*., 2005). The larvae were fed thrice daily, at 09.00, 13.00 and 17.00 hours. Each larva was given an approximate of 0.03 mg of a diet type. On emergence, the adults were offered 10% sugar solution soaked in cotton pads placed on the cages. The sugar solution served, as a source of energy. The energy sources were offered on a daily basis. Two days after emergence, the females were offered bovine blood provided via Hemotek® membrane feeding apparatus. The blood was obtained from a local abattoir and mixed with EDTA to prevent coagulation. On the second day after blood feeding, the females were provided with an oviposition dish to lay eggs. The eggs were then dispensed, larvae fed, larval water changed under similar conditions described by Yugi *et al*., (2014) as already stated.

### *2.4. Cow Dung Collection and Preparation*

Fresh cow dung was collected every morning between 08.00-08.30 hours from the College of Agriculture and Veterinary Sciences of the University of Nairobi, Kenya. The dung was sourced from a lactating seven year old hybrid (Hereford and Borana) cow. The cow and others grazed on kikuyu, star, oat and *Hyparhania* grass *spp.* within the college grazing grounds. The animals were later provided with hay from Rhodes and Sateria in the evening during milking. After collection, the dung was placed in a plastic bag and immediately transported to the laboratory at the School of Biological Sciences, University of Nairobi, Kenya for further processing and refrigeration at temperatures of 4$^{O}$C.

### *2.5. Larval Rearing Media Preparation*

Six larval rearing media of different pH (6, 7, 8 and 9), cow dung and clean untreated borehole water (pH 6.8) were used to rear L4s to pupation. All the media solutions contained 0.5 g of Potassium Hydrogen Carbonate ($KHCO_3$) except for solution of pH 6, clean borehole water and media made from cow dung. In particular, the rearing solutions were prepared as follows: (i) pH 6: two l of distilled water only, (ii) pH 7: 1 ml of 0.1 M hydrochloric acid and 0.5 g of $KHCO_3$ in 2 l of distilled water, (iii) pH 8: 0.5 g of $KHCO_3$ in 2 l of distilled water, (iv) pH 9: 0.5 Ml of 0.1M sodium hydroxide and 0.5 g of $KHCO_3$ in 2 l of distilled water, (v) Cow dung solution: 20 g of cow dung dissolved in 2 l of distilled water and (vi) clean borehole water: 2 l of untreated borehole water.

### *2.6. Microscope Calibration*

A light microscope (Unico) was calibrated to enable determination of mosquito sizes. This was done in the following manner. First, an ocular micrometer was mounted in one of the eyepiece lenses of the microscope followed by placement of a graticule slide on the stage of the microscope. Secondly, with the required objective lens in place (×40) the scale was adjusted until the zero line of the eyepiece scale aligned exactly with the zero line of the calibration scale on the graticule slide. This distance was 0.5 mm. The number of division between the distance between the two zero lines and where the next exact alignment between the ocular micrometer and graticule-slide scales occurred was also determined. This was 20µm. These measurements were then used to calculate the conversion factor with which direct wing length measurements were multiplied to estimate mosquito wing lengths. The conversion factor was calculated as follow:

$$\text{Conversion factor} = \frac{\text{Distance on graticule}}{\text{Divisions on ocular that matches graticule distance}}$$

$$\text{Conversion factor} = \frac{0.5\text{mm}}{20\mu\text{m}} = 0.025\text{mm}$$

1µm = 0.025 mm

*2.7.1. Eggs Hatching Rates of An. Arabiensis*

This experiment was conducted to determine the most suitable temperature regime (22 - 23°C or 28 - 29°C) for extended *An. arabiensis* egg storage period. The temperature regimes were settled on by slightly modifying the temperature regimes used in studies done by Meola, 1964 and Trpis 1972 on intraspecific variations in desiccation resistance by *Ae. aegypti* eggs. To facilitate this, *An. arabiensis* eggs were collected from *An. arabiensis* mosquitoes raised on different diet types (*Rastrineobola argentea*, Tetramin® Baby fish food and Bakers' active yeast) in their larval stages. The eggs were counted in two batches using fine tipped camel hair brushes under dissecting microscope (Leica Zoom 2000 at × 10 magnification) and placed in batches of 20s in paper towels in Petri dishes labeled with the date of preparation. One batch was placed on a table in a room maintained at 22 - 23°C and the other in a room maintained at 28 - 29°C. This procedure was repeated every two days 16 times to yield enough eggs to be stored for different period of time [zero days (eggs collected and used on the day of experiment), 2 days, 4 days, 6 days, 8 days, 10 days, 12 days, 14 days, 16 days, 18 days, 20 days, 22 days, 24 days, 26 days, 28 days, and 30 days].

On the experimental day, all the 32 sets of egg batches of 20 eggs each were dispensed each in separate conical oviposition cups. Each cup measured 4.0 cm top × 3.5 cm bottom × 2.7 cm height and contained 25 $cm^3$ of untreated borehole. Each cup was labeled with the temperature regime the eggs had been stored and length of time the eggs had spent in the room. The set ups were replicated five times (Table 1) and were left on tables in the insectary for two days to allow incubation and hatching.

**Table 1**. Experimental set up showing food type used to raise parental stock, eggs used per set up, number of replicates and total number of *An. arabiensis* eggs used per diet type

| Type of food | Number of eggs used per oviposition cup | | Replicates per day | Total number of eggs used |
|---|---|---|---|---|
| | 22 - 23°C | 28 - 29°C | | |
| Crushed silver cyprinid fish | 20 | 20 | 5 | 200 |
| TetraMin® fish food | 20 | 20 | 5 | 200 |
| Baker's yeast | 20 | 20 | 5 | 200 |

Forty-eight hours after the start of the experiment, the oviposition cups were carefully observed and larvae hatching from the eggs noted and counted. For cups where the number of hatched larvae were not equal to the number of dispensed eggs, all the unhatched eggs and shells were examined under a dissecting microscope and classified as either 'still viable but delayed in hatching' if it showed signs of hatching (eggs with open operculum) or 'non-viability' if it was non-hatched or with unopened opercula. The 'still viable' eggs were given more time to hatch. The numbers of larvae were recorded per temperature regime and diet type fed to the parental group at larvae stage and percentage mean egg hatchability determined using the formula;

$$\%\ \text{Mean egg hatchability} = \frac{\text{Number of counted L1}}{\text{Total number of eggs dispensed}} \times 100\%$$

*2.7.2. Pupation of An. arabiensis*

This experiment was conducted to determine the most suitable pH regime for expediting metamorphosis in mosquito production chain. Six sets of larval holding cups each measuring 7.5 cm top 5.0 cm bottom and 8 cm height consisting of ten larval holding cups were used. Each set of ten cups contained a particular rearing medium: (i) the first set contained solutions of pH 6, (ii) the second, pH 7, (iii) the third, pH 8, (iv) the fourth, solutions of fresh cow dung, (v) the fifth, pH 9 and (vi) the sixth untreated borehole water (pH 6.8). Each larval holding cup contained 100 Ml of the said larval medium.

Each day, a total of 60 L4s were randomly picked from the larval rearing trays and placed one in each cup for all the six sets of larval holding cups. Each larva was fed 0.03 mg of Tetramin® baby fish food per day. The experiments were started at 10.00 hours each day and allowed to continue until all the larvae pupated. The set ups were observed at an hourly interval from the start until 19.00 hours each day. The numbers of pupae developing were noted and recorded per the time duration taken for the L4 to pupate. The experiment was replicated five times. Any pupae that developed after 19.00 hours were reported to have developed after 24 hours. Every cup with a pupa was covered with mosquito netting and secured tightly at the top with a rubber band to prevent emerged adult from escaping. The pupae were left in their respective larval holding cups until the adults emerged. The sex of the emerged adult was determined by observing the antennae (male mosquitoes have highly feathered (plumose) antennae while female mosquitoes are sparsely feathered (pilose) antennae. A wing was removed from each emerged mosquito using a pair of fine tip forceps and measured to determine the size of the adult fly as described below.

*2.7.2.1. Estimating Mosquito Size*

Mosquito size was estimated by measuring the length of one of the mosquito wing (Zahiri and Rau, 1998). The wing was placed over a drop of water on a clean microscope slide, covered with a cover slip and then its length measured under a ×40 magnification from the distal end of alula to the tip, excluding the fringe scales.

*2.8. Statistical Analysis*

To study the effect of storage period, temperature and storage condition on hatching rate, correlation between percent hatch and storage period for each temperature/storage condition was tested and compared. The effects of temperature on egg viability and pH on pupation of L4s and the sex of emerging adult mosquitoes were analyzed as a function of mosquito size (based on wing length). Pearson correlation coefficient were calculated and tested for significance of each relationship at $p < 0.05$. Least-Squares regression lines were then determined and slopes and intercepts of lines were tested with Analysis of Variance in General Linear Model (Neter et al., 1996). All analyses were done using the Statistical Package for Social Scientists (SPSS) for windows version 11.5.

## 3. Results

### *3.1. Effects of Temperature and Storage Time on Egg Hatchability*

The experiment was conducted for a month. It was observed that egg hatchability was significantly reduced when stored at a higher temperature: eggs incubated at 28-29 °C were less hatchable than those incubated at 22-23 °C ($p < 0.05$). Egg hatchability was affected significantly by the duration of storage time ($p < 0.05$). Eggs, from mosquito parental stock raised on crushed silver cyprinid fish and TetraMin® baby fish food larval diets, were hatchable even after 8 days as opposed to 6 days for mosquitoes raised on baker's active yeast (Table 2). In all cases, egg hatchability reduced as the number of days progressed ceasing altogether after day eight for the best performing larval diet.

**Table 2.** Mean percentage of *Anopheles arabiensis* eggs hatching out of batches of 20 eggs kept under two different temperature regimes (22-23°C and 28-29°C)

| Time (days) | Crushed silver cyprinid fish | | Tetramin® baby fish food | | Baker's yeast | |
|---|---|---|---|---|---|---|
| | 22-23°C | 28-29°C | 22-23°C | 28-29°C | 22-23°C | 28-29°C |
| 0 | 71 | 64 | 68 | 24 | 71 | 70 |
| 2 | 12 | 31 | 62 | 15 | 20 | 10 |
| 4 | 13 | 0 | 13 | 0 | 12 | 0 |
| 6 | 7 | 0 | 8 | 0 | 1 | 0 |
| 8 | 4 | 0 | 1 | 0 | 0 | 0 |
| 10 | 0 | 0 | 0 | 0 | 0 | 0 |
| 12 | 0 | 0 | 0 | 0 | 0 | 0 |
| 14 | 0 | 0 | 0 | 0 | 0 | 0 |
| 16 | 0 | 0 | 0 | 0 | 0 | 0 |
| 18 | 0 | 0 | 0 | 0 | 0 | 0 |
| 20 | 0 | 0 | 0 | 0 | 0 | 0 |
| 22 | 0 | 0 | 0 | 0 | 0 | 0 |
| 24 | 0 | 0 | 0 | 0 | 0 | 0 |
| 26 | 0 | 0 | 0 | 0 | 0 | 0 |
| 28 | 0 | 0 | 0 | 0 | 0 | 0 |
| 30 | 0 | 0 | 0 | 0 | 0 | 0 |

### *3.2. Effects of pH on Pupation of L4s*

The experiment was conducted for ten days. It was found that the mean pupation time of L4 larvae maintained in untreated borehole water (pH 6.8) differed significantly compared to L4s maintained in other rearing media ($p < 0.05$) (Table 3). Mean pupation time was neither influenced by sex ($p = 0.124$) or size ($p = 0.801$) of emerged mosquitoes.

**Table 3.** Mean pupation time and pupation rates (proportion of larvae that pupated within eight hours) of L4s maintained in different rearing media. The proportion of eclosed male mosquitoes is shown in parenthesis

| Medium | N | Pupation time (h) | Pupation rate | Eclosion rate | Females | Wing size (mm) |
|---|---|---|---|---|---|---|
| Untreated borehole water (pH6.8) | 60 | 3.28 ± 0.25 [a] | 0.88 | 0.82 | 0.66 (0.34) | 3.12 ± 0.02[b] |
| Cow dung solution | 100 | 5.6 ± 0.47 [b] | 0.72 | 0.71 | 0.69 (0.31) | 2.96 ± 0.02[b] |
| PH 6 solution | 100 | 7.09 ± 0.62 [b] | 0.73 | 0.73 | 0.65 (0.35) | 2.97 ± 0.17[b] |
| PH 7 solution | 100 | 6.67 ± 0.44 [b] | 0.74 | 0.72 | 0.66 (0.34) | 3.03 ± 0.17[b] |
| PH 8 solution | 100 | 6.54 ± 0.63 [b] | 0.74 | 0.73 | 0.58 (0.42) | 2.99 ± 0.02[b] |
| PH 9 solution | 100 | 5.99 ± 0.47 [b] | 0.79 | 0.66 | 0.66 (0.34) | 2.98 ± 0.02[b] |

Notes:

1. Mean pupation time in hours of mosquito larval stages followed by different letter superscripts in the same row differ significantly.
2. Mean wing size in millimeters of adult mosquitoes followed by same letter superscripts in the same row do not differ significantly.

## 4. Discussion

In the present study, temperature and length of storage period as contributors to desiccation for mosquito eggs and pH as a determinant of rearing water quality for the larval stages of the mosquitoes were experimented on. The purpose of this step was to obtain information as to their contribution and mechanisms of manipulation towards optimizing mass rearing of the malaria vector *An. arabiensis* mosquitoes.

In the present study, it was found that higher temperatures were unfavorable to egg hatchability, an observation replicated by Bayoh and Lindsay (2003), Lyons *et al*., (2012) and Khan *et al*. (2013). Egg hatchability, in the present study, was found to be inversely proportional to storage time that is hatchability reduced with increased storage time a finding that was similarly reported for *An. gambiae* in Eritrea (Shililu *et al*., 2004).

*An. arabiensis* eggs in the present study remained viable for only 8 days. This time was similar to that reported for similar species (Khan *et al*., 2013) but shorter than 10 days reported for *An. gambiae* complex eggs kept in drying sandy loam (Shililu *et al*., 2004) and 12 days in dry soils (Beier *et al*., 1990). However, the observed dramatic reduction in the rate of egg hatchability especially after day zero (for freshly laid eggs) is consistent with that observed in other studies (Beier *et al*., 1990; Shililu *et al*., 2004; Khan *et al*., 2013).

There are many studies on the influence of temperature at the early developmental stages of *Anopheles gambiae* (Koenraadt *et al*., 2003), *Anopheles albitarsis*, *Anopheles*

*aquasalis* (Benedict, 1991), and *Aedes aegypti* (Farnesi *et al*., 2009). Indeed, microscopic observations on *An. gambiae* embryos showed that extreme high temperatures affect humidity that influences desiccation rates. This impacts normal mosquito egg development (Impoinvil *et al*., 2007). This might have been the case in the present study where a low rate of hatchability was observed for temperatures of 28-29$^{o}$C.

It is known that conditions experienced by juvenile mosquitoes determine mosquito adult characteristics (Lyimo *et al*., 1992; Beck-Johnson *et al*., 2013). In the present study the forth larval instars (L4s) were used to simulate the effect of pH on development and size of emerged adult *An. arabiensis* mosquitoes. Pupation was observed to be more rapid in untreated borehole water (pH 6.8) though mean pupation time for the different emerging sexes (male and female) were not affected by the pH of the rearing solution. The former findings differ while the latter agree with finding of Edillo *et al*. (2006) and Pelizza *et al*. (2007) who found no significant influence of pH on anopheline aquatic stages. Earlier however, water of near neutral pH (pH of 6.8 - 7.2) was observed to be most optimal for the weakening of the egg shells for the first instar larvae stage to emerge (Okogun *et al*., 2003). Indeed, *An. arabiensis* have been observed to associate with waters with low acidity (Robert *et al*., 1998), apparently via the use of ion exchange mechanisms, especially Na+/H+ exchangers, to move acid/base equivalents (Havas,1981). It is most probable that in the present study untreated borehole water (pH of 6.8) was less acidic and approximated the above conditions providing the most optimal condition for the weakening of L4s' and pupae exuvia leading to rapid pupation and emergence of adults mosquitoes, respectively.

Members of *An. gambiae* complex including *An. arabiensis* breed in clean shallow waters that are sunlit (McCrae, 1984; Kweka *et al*., 2012). These are the preferred optimal habitat conditions. In this study, the use of cow dung in the preparation of a rearing medium was to test if the mosquitoes could prefer otherwise and as the results showed this was the case as there was no significant effect of contamination on the development of *An. arabiensis* mosquitoes.

The present study concludes that pH did not contribute to the pupation rate of the L4s though near neutral pH (pH 6.8) was observed to be optimal for pupation. It is also noted that *An. arabiensis* eggs remained hatchable for 8 days when incubated under temperatures of 22-23$^{o}$C.

## Acknowledgements

I thank Baraza Sheila, Okumu Fred and Otieno-Ayayo, ZN for helping with the rearing of the experimental mosquitoes. Many thanks also go to International Atomic Energy Agency for providing *Anopheles arabiensis* eggs and funding this project (Research grant contract # KEN - 13291).

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JJBS
Jordan Journal of Biological Sciences

Volume 10, Number 1,March 2017
ISSN 1995-6673
Pages 13 - 18

# Culture Media Comparative Assessment of Common Fig (*Ficus carica* L.) and Carryover Effect

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*Received: October 20, 2016 Revised: January 3, 2017 Accepted: January 14, 2017*

**Abstract**

Production of common fig in Jordan has dropped more than 20-folds during the last five decades, due to several challenges, including biotic and abiotic stresses. The present study was conducted to asses a new protocol of shoot and callus development from fig apical buds. Explants of three local genotypes (Khdari, Mwazi and Zraki) were collected and grown in three culture establishment media: Murashige and Skoog (MS), Olive Medium (OM) and Woody Plant Medium (WPM). Two months later, shoot growth and callus development were measured, followed by transplanting into subculturing medium in test tubes with MS media. After additional two months, the same parameters were measured again. The results of the culture establishment showed that the highest shoot growth was obtained with OM and the highest callus development was obtained with WPM. On the other hand, the subculturing in MS medium showed prominent carryover effect of the first inoculation media, where the highest shoot growth was obtained in cultures transplanted from OM and the highest callus development was obtained in cultures transplanted from WPM. The present study delivers an improved protocol for establishment of common fig by tissue culture.

**Keywords**: Fig, in vitro, media, carryover effect.

## 1. Introduction

Common fig (*Ficus carica* L.) is one of the earliest cultivated plants. Fig fruits are known for their favorable taste and richness in nutrients and pharmacological compounds (Moon *et al*., 1997; Dhage *et al*., 2012). Common fig is native to the Mediterranean region, including Jordan (Sadder and Ateyyeh, 2006). However, the production of common fig in Jordan has dropped more than 20-folds during the last five decades. In addition, the production of common fig faces many challenges including biotic and a biotic stresses (drought, salinity, alkalinity, soil borne diseases and nematodes), (FAOSTAT, 2015). In Jordan, twelve local landraces are cultivated. Among which Khdari, Zraki and Mwazi are the most preferred and, together, they represent around 64% of the total cultivated fig in Jordan (Almugrabi and Anfoka, 2000). Increasing salinity has a negative effect on the number of shoot, shoot length and fresh and dry weight of common fig *in vetro* (Qrunfleh *et al.*). The number of fruits per shoot in the second crop of Khdari, Zraki and Mwazi are characterized by low, intermediate and high, respectively (Ateyyeh and Sadder, 2006b). The Zraki landrace produce the largest fig fruits in Jordan (up to 27.6 g) that are famous for their purple skin, while both Mwazi and Khdari have green fruit skin color (Ateyyeh and Sadder, 2006a).

Over the last few decades, tissue culture techniques have been used for rapid and large-scale propagation of a number of fruit trees (Bajaj, 1986; Zimmerman, 1986). The propagation by conventional method of (cuttings and grafting) is limited and slow. As those pieces can be obtained only from upright branches, which results in poor rooting and only 20–30% of the cuttings survive (Kumar *et al*., 1998).

*In vitro* culture of *Ficus* species has been widely studied as an alternative method for mass-scale production and high quality planting material (Rout *et al*., 2006). The successful results were obtained from using apical buds and shoots tip (Hepaksoy *et al*., 2006). The Murashige and Skoog (MS) (1962) culture medium is basically and widely used for plant tissue culture. Its components of salts are responsible for significant gains in tissue and cell development and growth. Additional media were also available for woody plants. The Woody Plant Medium (WPM) developed by Lloyd and McCown (1981) is the second most used medium for *in vitro* cultivation of woody species. It was developed for culturing shoots of woody plants and has found widespread use in the propagation of bushes and trees. WPM was shown to be beneficial for micropropagation of common fig (Brum, 2001; Mustafa *et al*., 2016). The olive medium (OM) is

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another medium developed for woody plants and utilized for micropropagation of olives (Rugini, 1984).

Most published articles of micropropagation of common fig focused on studying the effects of different concentrations of plant growth regulators on *in vitro* plant growth and development. However, the literature lacks comparative studies assessing multiple media. Therefore, we initiated this study to assess the effect of different media while ignoring the plant growth regulators' effects. Moreover, we are trying to move forward to build a strong tissue culture protocol for fig transplant production in Jordan.

## 2. Materials and methods

### *2.1. Explants Material*

Healthy shoot cuttings with apical buds were collected from fig trees, which were planted in Shafa Badran Agricultural Station, the University of Jordan (32°3'36"N 35°55'22"E). Around 60-70 shoot cuttings (15 cm long) were utilized from each landrace of Mwazi, Zraki, and Khdari. Cuttings were transported in water for 1 hour. Apical buds were excised from the shoot cuttings and directly submerged in citric acid solution (1.5 mg/l). The explants were surface-sterilized with absolute ethanol for one min. Thereafter, the buds were surface-sterilized with commercial bleach (1.625% sodium hypochlorite) and few drops of tween-20 (Sigma, USA) for 15 min with continuous shaking. The samples were washed with sterile distilled water three times.

### *2.2. Culture Media*

In the present study, three types of media were utilized, the first one was full strength MS medium (Murashige and Skoog, 1962), the second was WPM (Lloyd and McCown, 1981) and the third was OM (Rugini, 1984). In addition, all media contained 30 g/l sucrose and 1 m g/l 6-Benzylaminopurine (BAP) (Sigma, USA), the media were distributed in Petri dishes (20 ml each). The pH was adjusted in the range between (5.7-5.9) using 1 N NaOH and 1 N the HCl. The media were solidified with 1.2 g/l agar, and were sterilized by autoclaving at 121°C and 15 psi for 20 min.

### *2.3. Culture Establishment*

The buds were inoculated in dishes. Surface sterilized buds were cultured on the surface of the three media working under aseptic conditions in laminar flow hood. Cultures were maintained in the growth chamber under a daily photoperiod of 16/8 (light/dark) provided by cool white fluorescent light and 23±2°C for two months.

### *2.4. Subculturing*

After culture establishment, shoots and callus were transplanted to test tubes containing 10 ml MS medium for each tube in highly aseptic condition. The cultures were placed in the growth chamber under the same conditions used for culture establishment for additional two months.

### *2.5. Statistical Analysis*

For culture establishment, explants were planted *in vitro* in 7 dishes (replications); each dish containing three buds in a Completely Randomized Design (CRD). Same design was maintained for subculturing. Data were analyzed using SPSS version 22 (2013) statistical analysis program. Means were separated by Tukey HSD with $p < 0.05$.

The shoot and callus development were evaluated at the end of both culture establishment and subculturing stages. Growth scales were used to assess callus development for size (1 = < 5 $mm^2$, 2 = 5-10 $mm^2$, 3 = 10-15 $mm^2$, 4 = 15-20 $mm^2$, 5 = 20-25 $mm^2$) and shoot growth for height (1= < 1 cm, 2 = 1-2 cm, 3 = 2-3 cm, 4 = 3-4 cm, 5 = 4-5 cm).

## 3. Results

### *3.1. Culture Establishment*

Three different common fig landraces were used to obtain apical bud explants. Both callus development (Fig. 1.A) and shoot growth (Fig. 1.B) was achieved for the majority of cultures explants. Some cultures developed limited phenol oxidation in the medium surrounding the explant. However, it did not affect growth.

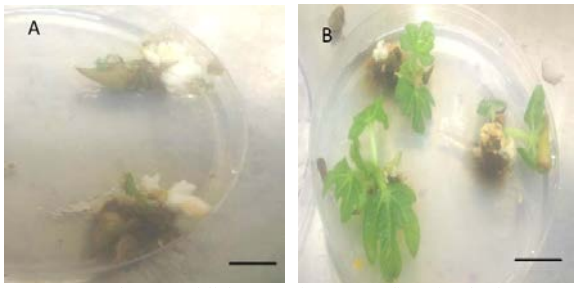


**Figure 1.** Culture establishment of common fig using bud explants 60 days old. A. Callus development in MS media, B. Shoot growth in WPM media, (Scale bar = 1 cm)

After two months of explant inoculation, when the data were combined, shoot growth in the landrace Khdari showed no significant differences between the three investigated media, whereas callus development was more prominent utilizing WPM when compared with the two other media (Table 1).For the Mwazi landrace, the highest significant shoot growth was achieved using OM compared to either MS or WPM. On the other hand, WPM gave the highest callus development compared to other media (Table 1). The shoot growth of the third landrace, Zraki, was not significantly different between the three investigated media, although the highest mean value was recorded for OM. The highest callus development in Zraki was achieved using WPM and the smallest was achieved using MS medium, while the OM resulted in an intermediate callus development which is not significantly different from those achieved by either WPM or MS medium (Table 1).

**Table (1)** Effect of different culture media on shooting and callus development of three different fig varieties namely; Khadri, Mwazi and zraki Means ± SD, Means followed by the different letters within the column are significantly different according to Tukey test at P ≤ 0.05

| | **Khdari** | | |
|---|---|---|---|
| Medium | **MS** | **OM** | **WPM** |
| Shoot growth | $1.5^{a}$±0.7 | $3.09^{a}$±0.7 | $2.2^{a}$±1.2 |
| Callus development | $1.5^{b}$±0.7 | $1.54^{b}$±0.7 | $3.0^{a}$±0.65 |
| | **Mwazi** | | |
| Medium | **MS** | **OM** | **WPM** |
| Shoot growth | $1.87^{b}$±1.12 | $3.33^{a}$±0.5 | $1.54^{b}$±0.68 |
| Callus development | $1.5^{b}$±0.75 | $1.0^{b}$±0.0 | $2.81^{a}$±0.75 |
| | **Zraki** | | |
| Medium | **MS** | **OM** | **WPM** |
| Shoot growth | $2.0^{a}$±1.0 | $3.5^{a}$±0.7 | $2.0^{a}$±0.86 |
| Callus development | $1.2^{b}$±0.44 | $1.5^{ab}$±0.7 | $2.22^{a}$±0.44 |

When the data was combined for all three common fig landraces, the significantly highest shoot growth was achieved for OM (Fig. 2), which is more than 30% greater than the shoot growth achieved by either of the remaining media. In contrast, the combined callus development data for all three landraces, showed significantly highest figure for explants cultures over WPM with almost one-fold increase as compared to either calli grown over MS medium or OM (Fig. 2).

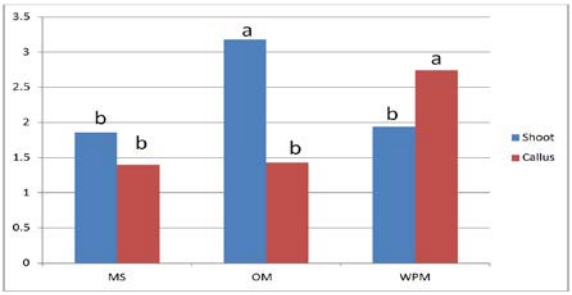


**Figure 2.** Effect of culture media on shoot and callus development of three different fig varieties of Jordan. Data represent means of all three landraces combined. Columns having different letters are significantly different

Furthermore, shoot growth showed no s ignificant differences when comparing between the three landraces using combined data of all media (Fig. 3). On the other hand, the landrace Khdari revealed the most prominent callus development from bud explants, while the smallest development was recorded for Zraki common fig landrace (Fig. 3)

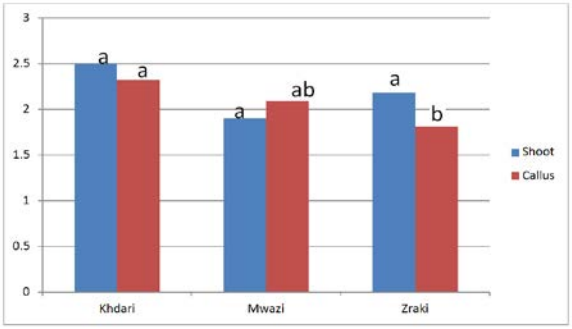


**Figure 3.** Effect of common fig landrace on shoot growth and callus development (Data represents means of all three culture media combined). Columns having similar letters are significantly not different

### *3.2. Subculturing*

After culture establishment, the same explants were further transplanted into fresh MS medium to test the carryover effect of various culture media (Fig. 4). After a while the old shoots started to become discolored and died, however, new shoots grew from the bud base and those were considered for data measurement.

**Figure 4.** Shoot and Callus development of Mwazi buds after 60 days of .subculture. (Scale bar = 1 cm)

Two more months later, shoot growth in the landrace Khdari was highest in cultures transplanted from OM followed by WPM and then MS medium, with 3.5, 2.75 and 1.5, respectively (Table 2). Meanwhile, callus development was more progressive in cultures transplanted from WPM with at least 1.5-fold increase compared to the two other media. The shoot growth in the landrace Mwazi was highest in cultures transplanted from OM followed by MS medium and then WPM, with 4.12, 2.9 and 2.18, respectively (Table 2). On the other hand, there were no significant differences for callus development for cultures transplanted from any media. Likewise, Zraki shoot growth was highest in cultures transplanted from OM followed by MS medium and then WPM, with 3.6, 2.33 and 2.09, respectively (Table 2). The highest callus development in Zraki was achieved in cultures transplanted from WPM with more than 2-fold increase compared to cultures transplanted from either MS medium or OM.

**Table (2)** Media carryover effect on shoot growth and callus development of transplanted cultures. Means ± SD, Means followed by the different letters within the column are significantly different according to Tukey test at P ≤ 0.05

| | **Khdari** | | |
|---|---|---|---|
| Previous medium | **MS** | **OM** | **WPM** |
| Shoot growth | $1.25^{b}$±0.35 | $3.5^{a}$±1.18 | $2.75^{ab}$±0.65 |
| Callus development | $1.5^{b}$±0.7 | $1.33^{b}$±0.51 | $3.87^{a}$±1.12 |
| | **Mwazi** | | |
| Previous medium | **MS** | **OM** | **WPM** |
| Shoot growth | $2.9^{ab}$±0.87 | $4.12^{a}$±0.62 | $2.18^{b}$±1.13 |
| Callus development | $2.2^{a}$±1.03 | $1.5^{a}$±0.57 | $2.12^{a}$±083 |
| | **Zraki** | | |
| Previous medium | **MS** | **OM** | **WPM** |
| Shoot growth | $2.33^{b}$±0.75 | $3.60^{a}$±0.54 | $2.09^{b}$±0.7 |
| Callus development | $1.33^{b}$±0.51 | $1.1^{b}$±0.22 | $3.09^{a}$±0.7 |

When the data were combined again for all three common fig landraces, the significantly highest shoot growth was achieved for cultures transplanted from OM (Fig. 5), which is this time around 40% greater than the shoot growth achieved from cultures transplanted from by either of the remaining media. In contrast, the combined callus development data for all three landraces showed significantly highest figure for explants cultures transplanted from WPM with almost one-fold increase as compared to either calli grown over MS medium or OM (Fig. 5).

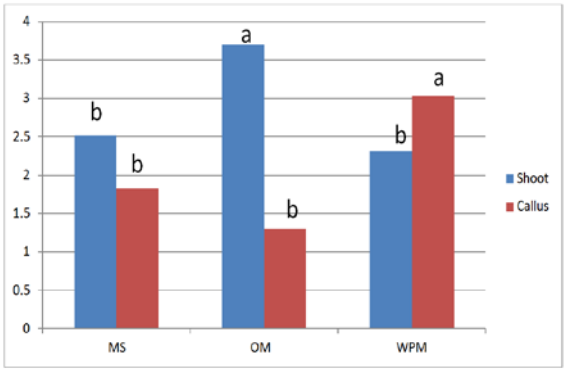


**Figure 5.** Effect of culture media before subculture on shoot growth and callus development (Data represents means of all three landraces combined). Columns having similar letters are significantly not different

Moreover, shoot growth showed no s ignificant differences when comparing between the three landraces using combined data from cultures transplanted from all media (Fig. 6). Likewise, no significant differences were recorded between common fig landraces for callus development from bud explants transplanted from all cultures (Fig. 6).

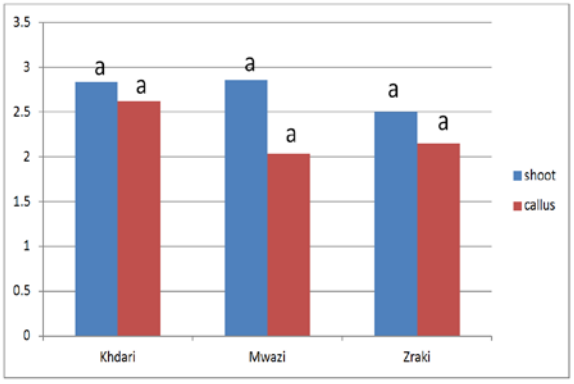


**Figure 6.** Effect of common fig landrace on shoot growth and callus development (Data represents means of cultures transplanted from all three media combined). Columns having similar letters are significantly not different

## 4. Discussion

When comparing the results of culture establishment data (Fig. 2) with culture subculture data (Fig. 5) after transplanting the cultures,the highest means of shoot growth was obtained in OM (3.18) and (3.7) for establishment and subculture, respectively ,and in the same time the highest means of callus development was clear by using WPM (2.74) and (3.03)for establishment and subculture, respectively. Similar trends can be found, emphasizing the carryover effect of initial culture medium. This is clear for shoot growth, which was outstanding in OM during culture establishment and after transplanting to new medium from OM. Similarly, callus development was outstanding in WPM during culture establishment and after transplanting to new medium from WPM.

MS, Knudson, WPM, and White and B5 media were assessed for micropropagation of common fig (Brum, 2001). Each medium was tested with four levels of sucrose (0, 15, 30 and 45 g/l). The results indicated that WPM supplemented with 20 g sucrose gave a high number of shoots and excellent growth of roots. Consequently, other reports have recommended WPM for common fig micropropagation (Fráguas *et al*., 2004a; Fráguas *et al*., 2004b; Mustafa *et al*., 2016).

However, our results (Figs. 2 and 5) show that OM is a much better alternative to WPM. In olives, highest shoot regeneration was achieved from OM medium supplemented with thidiazuron or zeatin (Rugini, 1984; Mencuccini and Rugini, 1993; Zacchani and De Agazion, 2004). Unfortunately, only limited initiatives have assessed the potential of OM for woody plants other than olives. Shoot establishment and proliferation were achieved in guava cultured over OM (Papadatou *et al*., 1990). In addition, OM was found to stimulate the development of new branches in *Juniperus phoenicea* (Loureiro *et al*., 2007).

Our comparative investigation of MS medium, OM and WPM is basically a comparison of relative concentrations of macro- and micro-element as correlated to *in vitro* establishment of common fig. Important differences can be noticed when comparing the element concentrations in OM (Rugini, 1984), WPM (Lloyd and McCown, 1981) and MS medium (Murashige and Skoog, 1962). Boric acid concentration is almost twice in OM (12.2 mg/l) compared to either WPM or MS medium (6.2 mg/l). Boron (B), complexes with mannitol, mannan, polymannuronic acid, and other constituents of cell walls, is involved in cell elongation and nucleic acid metabolism (Taiz and Zeiger, 2002). Furthermore, magnesium sulfate concentration is four times higher in OM (732 mg/l) compared to both of MS and WPM (180.7 mg/l). Magnesium is required by many enzymes involved in phosphate transfer and is a major constituent of the chlorophyll molecule (Taiz and Zeiger, 2002). Moreover, potassium phosphate (Monobasic) concentration in OM (340 mg/l) is twice the level in either MS medium or WPM (170 mg/l). Phosphorus is component of sugar phosphates, nucleic acids, nucleotides, coenzymes, phospholipids and phytic acid. It has a k ey role in energy transfer reactions (Taiz and Zeiger, 2002). Another major difference is the higher concentration of zinc sulfate (Heptahydrated) in OM (14.3 mg/l) compare with the other two media (8.6 mg/l). Zn is a constituent of alcohol dehydrogenase, glutamic dehydrogenase, carbonic anhydrase (Taiz and Zeiger, 2002). Therefore, such elevated concentrations of these various elements in OM would be more beneficial in fulfilling shoot growth requirements in common fig as compared to WPM or MS medium.

Our data show that the WPM media gave the highest callus production in all common fig landraces investigated

in this study (Khdari, Zraki and Mwazi). This effect was further emphasized by the carryover effect on callus development of cultures transplanted from WPM compared to the other two media. This would be also promoted by the BAP supplied in our medium. BAP is one form of routinely applied cytokinins in plant micropropagation. They promote division, elongation and differentiation, delaying plant senescence, promote the breaking of the apical dominance and induce proliferation of axillary shoots (Taiz and Zeiger, 2002). Although BAP is the most widely used cytokinin, this does not mean it is ideal for all species. Similar to our results, callus development was found to be promoted in common fig using WPM supplemented with BAP (Fráguas *et al*., 2004b). The use of kinetin in the culture medium decreased the formation of callus in common fig (Jordan and Iturriaga, 1980). However, good callus induction was shown in the MS medium supplemented with 0.4 mg/l kinetin and 4.0 mg/l 2, 4-D (Danial *et al*., 2014). On the other hand, callus development is concentration dependent, fresh and dry callus weights were increased linearly with the increasing kinetin concentrations (Fráguas *et al*., 2004a).

Callus induction by WPM was also reported for other plant species, such as in *Barringtonia racemosa* (Behbahani *et al*., 2011) when compared with cultures grown over other media, like MS and B5. Furthermore, a fast callus growth was achieved by WPM medium with 2, 4-D. Farzinebrahimi *et al*. (2014) also found high percentage of callus formation with best dry and fresh weights to be formed on WPM supplemented with 2,4-D and NAA in *Gardenia jasminoides* Ellis. In fact, the concentration of various elements of three media investigated in our study, we can notice an elevated concentration of manganese sulfate in WPM (22.3 mg/l) as compared to both OM and MS medium (16.9 mg/l). In addition, Thiamine-HCl levels are 1.0, 0.1 and 0.5 mg/l in WPM, MS medium and OM, respectively. These important constituents with elevated levels in WPM may explain the promoting ability for callus development more than the other media. Thiamine was recorded to be essential for callus formation in other plant species including soybean (Ikeda *et al*., 1976) and date palm (Al-Kayri, 2001).

## 5. Conclusion

Our results revealed the importance of the medium per se and how much it could affect shoot growth and callus development. The data showed that the OM is more suitable for shoot growth of apical bud in common fig than either WPM or MS medium. On the other hand, WPM media were found to be crucial for callus induction and subculturing for different fig landraces. Moreover, it is important to consider the carryover effect of initial inoculation medium on both subsequent tissue growth and development.

## 6. Acknowledgment

The authors would like to thank the Deanship of Academic Research at the University of Jordan for funding this research.

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JJBS
Jordan Journal of Biological Sciences

Volume 10, Number 1,March 2017
ISSN 1995-6673
Pages 19 - 28

# Cryopreservation of *Thymbra spicata* L. var. *spicata* and Genetic Stability Assessment of the Cryopreserved Shoot Tips after Conservation

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*Received: November 4, 2016 Revised: January 3, 2017 Accepted: January 14, 2017*

**Abstract**

The present study aims to introduce endangered Thymbra spicata L. to cryopreservation and to assess genetic stability of shoot tips after cryopreservation using Amplified Fragment Length Polymorphism (AFLP) technique. For the cryopreservation experiments, vitrification and droplet vitrification techniques were examined. In the first vitrification experiment, highest survival (80%) and regrowth (35%) rates were obtained in shoot tips exposed to 2- stepwise application of Plant Vitrification Solution (PVS) technique. Results of the second experiment showed that, 60 min. was the best duration for loading the explants with the loading solution before exposure to PVS2 as it yielded the highest survival and regrowth rates ( 85 , 45% ) after cryopreservation. Moreover, in the third experiment, 2 M glycerol + 0.4 M sucrose had proven to be the best formula as a loading solution compared to the results obtained in the other combinations. Additionally, in the fourth experiment, the highest survival and regrowth percentages (75, 35%) were obtained in explants treated with PVS2 compared to results obtained in tips treated with other PVS solution types. In droplet vitrification experiment, the highest survival and regrowth rates (80, 35%) were recorded in explants exposed to PVS2 for (45 min.). Meanwhile, no genetic differences were detected in the shoot tips before and after cryopreservation.

**Keywords**: : Cryopreservation, Genetic stability, Droplet- vitrification, Vitrification.

## 1. Introduction

Cryopreservation protocols were successfully used to conserve many plants for indefinite period of time (Vasanth and Vivier, 2011). Additionally, maintenance of genetic stability of plant material is another achievement of cryopreservation beside long term conservation, and the validity of cryopreservation is only achieved when genetic stability is unchanged after exposure to liquid nitrogen (Zarghami et al., 2008). For decades, it was believed that as the explant is introduced to cryogenic storage, this would cease all forms of metabolic activities, which keeps the plant material true to type after storage (Panis et al., 2002; Kaczmarczyk et al., 2012). Recently, evidence of genetic alterations after cryopreservation of in vitro-derived plant material has been reported in some studies, and, consequently, evaluation of plant genetic uniformity has been carried out to validate newly established cryopreservation protocols (Harding, 2004; Micula et al,, 2011). In many instances, the genetic alteration in the cryopreserved specimens was reported to be accidental, most frequently was carried on a single specimen and was characterized by few markers (Martin and Gonzalez-

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Benito, 2005; Sanchez et al., 2008; Micula et al., 2011). Therefore, there is a growing interest in the assessment of plant genetic uniformity to insure the fidelity of the plants after their exposure to cryopreservation techniques (Harding, 2004; Micula et al., 2011).

Examples on different genotypes that were reported with a genetic variation after cryopreservation are Malus pumila (Hao et al., 2001), Carica papaya (Kaity et al., 2008), Fragaria gracilis (Hao et al., 2002a), Citrus sinensis (Hao et al., 2002b), Ribes sp. (Johnston et al., 2009) and Prunus dulcis (Channuntapipat et al., 2003).

Thymbra spicata L. var. spicata is a medicinal plant that grows wild in the Mediterranean region including Jordan (Akine et al., 2010; Inan et al., 2011; Royal Botanic Garden (RBG) 2012). This plant is used as a spice and as an herbal tea for treating asthma, colic, bronchitis and coughs (Baser, 2002; Baydar et al., 2004). Moreover, Thymbra spicata L. var. spicata has remarkable antifungal, antibacterial and antimicrobial activities that resulted from "carvacrol", the major chemical component of this plant (Akine et al., 2010; Inan et al., 2011). Meanwhile, Thymbra spicata L. var. spicata is facing threats in Jordan due to the uncontrolled collection in addition to the extensive greasing by animals (Taifour and Al-Oqlah, 2014).

In the present study, *Thymbra spicata* L. var. *spicata* was introduced to cryogenic storage using vitrification and droplet vitrification techniques, and genetic stability of the shoot tips was proved to be maintained after cryopreservation.

## 2. Materials and methods

### *2.1. In vitro Establishment of Thymbra spicata* L. *var. spicata*

The plant material was supplemented by the Royal Botanic Garden (RBG) (Tell Al-Rumman, Jordan) in a form of mature seeds collected on September 2012 from a single mother plant growing solely at RBG located between 32.18772° Latitude and 35.827393° Longitude. Mature seeds of *Thymbra spicata* L. var. *spicata* were surface-sterilized by washing under running tap water for 20 min. Seeds were then immersed in 20% sodium hypochlorite for 10 min before three times rinsing (5min/ time) with sterile distilled water under laminar air-flow. After that, seeds were soaked in 70% ethanol (v/v) for 30 seconds before being rinsed with sterile distilled water for three times (5min. each). Sterilized seeds were then cultured in 100 ml bottles containing Water Media (WM) (water and 0.8 %) supplemented with ($2.9*10^{-6}$ M) gibberellic acid (GA3). Seeds were maintained in growth room under a daily regime of 24±1 °C under a 16/8 (light/dark) photoperiod of 45–50 µmol m−2s−1 irradiance, until germination.

The germinated seedlings were directly subcultured onto the prepared proliferation media consisted of a full strength MS media (Murashige and Skoog, 1962) supplemented with 0.1 M sucrose, $2.7*10^{-6}$ M Benzylaminopurine (BAP) and $9.8*10^{-8}$ M Indolebutric Acid (IBA). The proliferated microshoots were subcultured into the same proliferation media every 4 weeks and cultures were maintained in the growth room under a daily regime of 16- hr light, 8- hrs dark and 24 ± 1°C.

### *2.2. In vitro Conservation Using Vitrification Technique*

All vitrification experiments in section 2.2 were carried out following the protocol stated by Rabba'a et al. (2012).

#### *2.2.1. Effect of Stepwise Application of Plant Vitrification Solution (PVS2)*

Shoot-tips of *Thymbra spicata* L. var. *spicata* were disected and precultured aseptically into a hormone free MS solid medium supplemented with 0.3 M sucrose for 3 days under dark. Then shoot-tips were transferred to sterile cryovials before being loaded with 1.0 ml loading solution (liquid- hormone free MS medium + 2 M glycerol + 0.4 M sucrose) for 20 min at 25 ºC. Next, the loading solution was removed using a sterile micropipette and replaced with 0.8 ml of PVS2 (liquid hormone free-MS medium + 30% glycerol + 15% Ethylene Glycol (EG) + 15% Dimythelsulfoxide (DMSO) + 0.4 M sucrose) for 20 min in three different methods.

1. Shoot-tips were exposed to a full strengh PVS2 for 20 min at 25 ºC, then half of the treated cryovials was plunged directly in LN (Liquid Nitrogen) for 1 hr while the other half was not exposed to LN.
2. Shoot-tips were exposed to 60% PVS2 for 10 min, followed by 100% PVS2 for another 10 min at 25 ºC, and then half of the treated cryovials was plunged directly in LN for 1 hr while the other half was not exposed to LN.
3. Shoot-tips were exposed to 20, 40, 60 and 100% for 20 min (5 min for each concentration) at 25 ºC, then half of the treated cryovials was plunged directly in LN for 1 hr while the other half was not exposed to LN.

After each treatment, PVS2 solution was removed and the non-cryopreserved shoot tips (-LN) were unloaded by unloading solution (liquid hormone free-MS medium + 1.2 M sucrose) washed 3 times (10 min/ time) before being transferred to a recovery solid hormone free-MS media supplemented with 0.1 M sucrose and kept under dark for 4 days, then transferred to dim light (20 µmol $m^{-2}s^{-1}$ irradiance) for another 4 days before being transferred to normal light conditions. After three weeks, shoot-tips were examined under the binocular microscope for any regrowth signs. After cryogenic exposure (+LN), cryovials were thawed in a water bath at 38 ºC for 2-3 min and then treated with the unloading solution (liquid hormone free-MS medium + 1.2 M sucrose) washed 3 times (10 min/ time) before being transferred to a recovery solid hormone free-MS media supplemented with 0.1 M sucrose and then stored under same conditions described above. After three weeks shoot-tips were examined under the binocular microscope for any regrowth signs. Survival percentage was recorded by testing shoot tips from each of +LN and –LN treatments using TTC (2, 3, 5-triphenyl tetrazolium chloride) which was prepared by dissolving TTC salt (0.5% (w/v) in 50 mM $K_2HPO_4$ at pH 7.0 after 15 h incubation at 25 ºC under dark. Survival percentage was recorded by testing shoot tips from each of treatment using TTC (2, 3, 5-triphenyl tetrazolium chloride) test. Survival percentages were calculated according to the following formula:

Survival percentage = [number of shoots with red color that result due to hydrogen ions released from cells respiration of viable cells) /total number of shoots] × 100%.

*2.2.2. Effect of Loading Solution Type*

The shoot-tips were precultured, as described in section 2.2.1, and then placed in cryovials before being divided into 3 parts. Each part was loaded with (1.0 ml) of one of the following loading solutions:

1. Hormone- free liquid medium + 1.0 M sucrose for 20 min at 25 ºC.
2. Hormone- free liquid medium + 2.0 M glycerol + 0.4 M sucrose for 20 min at 25 ºC.
3. DMSO (5 or 10%) + sucrose (0. 5 or 0.75 M) for 20 min at 25 ºC.

Next, the vitrification solution PVS2 was removed and the non-cryopreserved (-LN) shoot tips were unloaded and subcultured, as described in section 2.2.1. Then shoot tips were examined after three weeks for any regrowth sign.

After cryopreservation (+LN), shoot tips were thawed, washed and subcultured, as described in section 2.2.1 before being tested after three weeks shoot for any regrowth sign. Survival percentage was recorded by testing shoot tips from each of +LN and –LN treatments using TTC (2, 3, 5-triphenyl tetrazolium chloride) assay as described in section 2.2.1.

*2.2.3. Effect of Loading Duration of the Loading Solution*

*S*hoot-tips were precultured as described in section 2.2.1 then placed in cryovials before be ing loaded with 1ml of loading solution (2.0 M glycerol plus 0.4 M sucrose in liquid hormone free-MS medium) for different periods of time (0, 10, 20, 40, 60 or 80 min) at 25 ºC. Next, the loading solution was replaced with 1.0 ml of 100% PVS2 at 25 ºC.

PVS2 solution was then removed and the non-cryopreserved shoot tips (-LN) were unl oaded and subcultured as described in section 2.2.1., then regrowth percentage was recorded after three weeks. Shoot tips that were exposed to LN were thawed, washed and subcultured, as described in section 2.2.1 and regrowth data were taken after three weeks.

Survival percentage was recorded by testing shoot tips from each of +LN and –LN treatments using TTC (2, 3, 5-triphenyl tetrazolium chloride) assay, as described in section 2.2.1.

*2.2.4. Effect of Plant Vitrification Solution (PVS) Type*

*Thymbra spicata* L. var. *spicata* shoot-tips were precultured, as described in section 2.2.1, then placed in cryovials before be ing divided into 3 parts and each part was loaded with (1.0 ml) of one of the following loading solutions:

1. Hormone free-liquid medium + 2.0 M glycerol + 0.4 M sucrose for 20 min at 25 ºC.
2. Hormone free-liquid medium + DMSO (10%) + 0. 5 M sucrose for 20 min at 25 ºC.
3. Hormine free-liquid medium + DMSO (10%) + 0.75 M sucrose for 20 min at 25 ºC.

The loading solution was then replaced with 0.8 ml from one of the following vitrification solution:

1. PVS2 (liquid hormone free-MS medium + 30% glycerol + 15% ethylene glycol (EG) + 15% dimythelsulfoxide (DMSO) + 0.4 M sucrose) at 25 ºC for 20 min.
2. Liquid hormone free- MS media + 15% DMSO and 1.0 M sucrose at 25 ºC for 20 min.
3. Liquid hormone free- MS media + 30% DMSO and 1.0 M sucrose at 25 ºC for 20 min.
4. PVS3 (liquid hormone free- MS media + 40% glycerol + 40% sucrose) at 25 ºC for 20 min.

PVS2 solution was removed and the non-cryopreserved shoot tips were unloaded and subcultured as described in section 2.2.1., then after three weeks, regrowth percentage was recorded.

For the cryopreserved shoot tips, the cryovials were thawed, washed and subcultured, as described in section 2.2.1., and regrowth data were taken after three weeks.

Survival percentage was recorded by testing shoot tips from each of +LN and –LN treatments using TTC (2, 3, 5-triphenyl tetrazolium chloride) assay, as described in section 2.2.1.

*2.3. In vitro Conservation Using Droplet Vitrification Technique*

Shoot tips of *Thymbra spicata* L.var. *spicata* were isolated aseptically then precultured, as described in section 2.2.1. Next, the pretreated shoot tips were loaded with the loading solution at consisted of (hormone free-MS liquid medium + 2 M glycerol + 0.4 M sucrose) at 25 ºC for 20 m in then the exposed to chilled PVS2 (hormone free-MS liquid medium + 30% glycerol + 15% Ethylene Glycol (EG) + 15% Dimythelsulfoxide (DMSO) + 0.4 M sucrose) for different periods of time (0, 10, 20, 35 and 45 min) at 0 ºC. The shoot tips were then put on sterilized aluminum foil strips (four shoot tips / strip). A single drop of chilled PVS2 was dripped onto each shoot tip, then half of the treated shoot tips were put into a cryovial and immersed into liquid nitrogen while rest of the shoot tips were directly rinsed with unloading solution (MS hormone free liquid media + 1.2 M sucrose) 3 times (10 min/time) at room temperature. For the cryopreserved shoot tips, after exposure to liquid nitrogen, cryovials were thawed in a water bath at 38 ºC for 2-3 min, and the aluminum foil was taken out from the cryovial, and rinsed with unloading solution (MS hormone free liquid media + 1.2 M sucrose) 3 times (10 min/time) at room temperature.

After unloading, the cryopreseved and non-cryopreserved shoot tips were thawed, washed and subcultured, as described in section 2.2.1 and regrowth data were taken after three weeks.

Survival percentage was recorded by testing shoot tips from each of +LN and –LN treatments using TTC (2, 3, 5-triphenyl tetrazolium chloride) assay, as described in section 2.2.1.

*2.4. Assessment of Genetic Stability Using Amplified Fragment Length Polymorphism (AFLP) Technique*

To assure the genetic uniformity of tested material, a single microshoot was propagated in the *in vitro* proliferation media described above and then used in all cryopreservation experiments, as described above. Shoot tips recovered before and after cryopreservation were assessed for their genetic stability using Amplified

Fragment Length Polymorphism (AFLP) technique. Also, two samples taken from the wild mother plant and another seed tissue cultured originated microshoot were included in the genetic assessment in order to compare them with the results obtained from the cryopreserved shoot tips.

Extraction of *Thymbra spicata* L. var. *spicata* genomic DNA was performed using the DNeasy Plant Mini Kit (Qiagene, Germany). For AFLP analysis, *Mse*I unlabeled primers, shown in (Table 1), were used following the procedure of Vos *et al.* (1995). The separation of the AFLP products (3µl for each sample) was performed in a 6.5% denaturing polyacrylamide sequencing gel (KBPlus 6.5% gel, LI-COR) with 0.5 × TBE electrophoresis buffer using the LI-COR DNA Analyzer (Model 4300 DNA Analyzer, LI-COR, Lincoln NE, USA) following the following the manufacturer's instructions. The gel was scanned on the LI-COR Odyssey® Infrared Imaging System (LI-COR, Lincoln NE, USA) and the resulted bands were then scored manually for the presence or absence in each sample.

**Table 1:** *Mse*I unlabeled primers used for AFLP analysis

| *Mse*I unlabeled primers |
|---|
| M-CTT + E-ACT (IRDye 700), M-CTA + E-ACT (IRDye 700) |
| M-CTC + E-ACT (IRDye 700), M-CAG + E-ACT (IRDye 700), |
| M-CTT + E-AAC (IRDye 800), M-CTA + E-AAC (IRDye 800), |
| M-CTC + E-AAC (IRDye 800 |
| M-CAG + E-AAC (IRDye 800) |

### *2.5. Experimental Design*

All treatments were arranged in a Completely Randomized Design (CRD). Each treatment consisted of five replicates with four explants/ replicate. The collected data were statistically analyzed using Statistical Package for the Social Sciences (SPSS) and standard error was calculated for the cryopreservation treatments.

## 3. Results

### *3.1. In vitro Conservation Using Vitrification Technique*

#### *3.1.1. Effect of Stepwise Application of Plant Vitrification Solution (PVS2)*

The results for none-cryopreserved shoot tips of *Thymbra spicata* L. var. *spicata* showed that stepwise application of PVS2 did not affect shoot tips survival or regrowth after exposure to PVS2 as tetrazollium test had revealed a full survival and regrowth of the treated shoot tips (Table 2). On the other hand, data showed that using 2-step application method of PVS2 (60% PVS2 for 10 min followed by 100% PVS2 for 10 min) had improved survival and regrowth rates of the shoot tips after cryopreservation and recorded the highest survival and regrowth rates (80% and 35%), respectively compared to the results obtained when full strength PVS2 was applied to the shoot tips as one shot (Table 2, Figure 1). Meanwhile, 4-step wise application of PVS2 did improve neither survival nor regrowth in *Thmbra spicata* L. var. *spicata* shoot tips after exposure to LN (Table 2)

Direct exposure of explants to full strength PVS2 was found to reduce recovery in many plants before and after cryopreservation, such as in shoot tips of potato (Kaczemarczyk *et al.*, 2008) and grapevine, which was attributed to the toxic nature of PVS2, as it is composed of highly toxic and concentrated ingredients which are applied to promote cell dehydration before exposure to LN (Markovic *et al.*, 2013). Also, many related studies were in full agreement with the obtained results, as they reported two- stepwise application of PVS2 as a proper measure to improve survival and regrowth in some plants such as *Phoenix dactylefera* (Subaih *et al*., 2007), *Artemisia herba- alba* (Sharaf, 2010) and *Achelliae fragrantissima* (Younis, 2012). On the other hand, Mogs *et al*. (2004) reported no significant effect of stepwise application of PVS2 on recovery rates of *Samipoulia ionanth* shoot tips after cryogenic conservation.

**Table 2:** Survival and regrowth percentages of vitrified shoot tips of *Thymbra spicata* L. var. *spicata* as affected by stepwise application of plant vitrification solution PVS2

| Non- cryopreserved (-LN) | | | Cryopreserved (+LN) | | |
|---|---|---|---|---|---|
| PVS2 concentration (%) | Survival % | Regrowth % | PVS2 concentration (%) | Survival % | Regrowth % |
| 20-40-60-100 | 100.0 ± 0.0* | 100.0 ± 0.0 | 20-40-60-100 | 60.0 ± 6.1 | 10.0 ± 1.0 |
| 60-100 | 100.0 ± 0.0 | 100.0 ± 0.0 | 60-100 | 80.0 ± 9.3 | 35.00 ± 9.0 |
| 100 | 100.0 ± 0.0 | 95.0 ± 5. 0 | 100 | 20.0 ± 6.2 | 5.0 ±0.4 |

Values represent percentages ± standard error

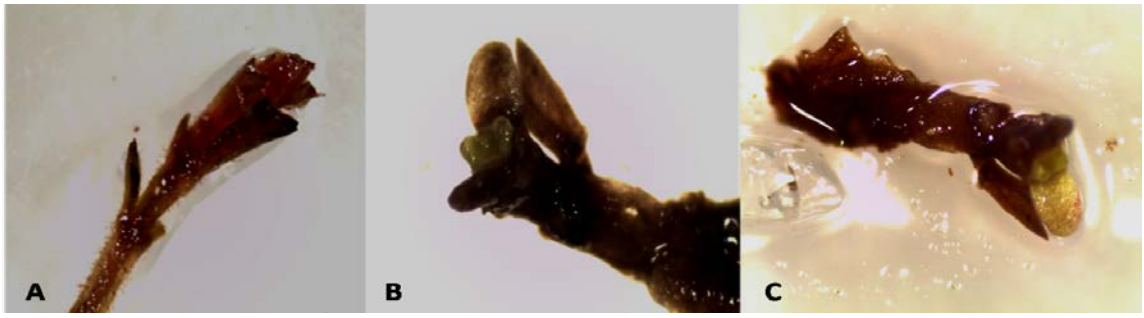


**Figure 1.** Survival and regrowth of vitrified and cryopreserved shoot tips of *Thymbra spicata* L. var. *spicata* using 2- stepwise application of PVS2 solution method (60% PVS2 for 10 min followed by 100% PVS2 for 10 min). A: Survival using TTC test, B: Start of regrowth after 3 weeks, C: Regrowth after 8 weeks

*3.1.2. Effect of Loading Duration*

In the second vitrification experiment, data showed that the survival and regrowth rates had decreased slightly when shoot tips of *Thymbra spicata* L. var. *spicata* were not loaded with the loading solution before exposure to PVS2 in the non-cryopreserved shoot tips compared to the other treatments (Table 3). On the other hand, the effect of loading duration of the loading solution had significantly affected both survival and regrowth of after plunging in LN (Table 3). The highest survival (85%) and regrowth (45%) rates were recorded in cryopreserved shoot tips treated with the loading solution for (60 min) (Table 3, Figure 2). Meanwhile, when exposure time to the loading solution exceeded or was less than (60 min), the survival and regrowth rates were adversely affected (Table 3). Sakai and Engelmann (2007) reported that treating the explants before cryopreservation with the Loading Solution (LS) was an effective measure to improve plant cell resistance against the toxic effect of PVS2. However, the survival and regrowth rates in *Thymbra spicata* L. var. *spicata* decreased as exposure durations exceeded or were was less than (60 min). This might indicate that when loading durations to the loading solution were short this could be insufficient for optimum protection of the plant cell against PVS2 toxic hazard and/ or to remove the intracellular water that could have resulted in intracellular ice crystallization after exposure the LN (Al-Ababneh *et al*., 2002; Subaih *et al*., 2007; Kaviani, 2011). Also, the obtained results were in full agreement with Shatnawi *et al.* (2011) who reported a 20%-regrowth in cryopreseved shoot tips of *Caperers spinoza* after 60 min exposure to the loading solution. On the other hand, Mogs *et al.* (2004) reported that exposing African violet for 10-20 min to the loading solution was enough to improve the survival and regrowth after cryopreservation which might indicate that the loading duration is plant-species-dependent.

**Table 3:** Survival and regrowth percentages of vitrified shoot tips of *Thymbra spicata* L. var. *spicata* as affected by different loading solution exposure durations

| Non- cryopreserved (-LN) | | | Cryopreserved (+LN) | | |
|---|---|---|---|---|---|
| Duration (min.) | Survival % | Regrowth % | PVS2 concentration (%) | Survival % | Regrowth % |
| 0.0 | 90.0 ± 6.1* | 65.0 ± 12.10 | 0.0 | 0.0 ± 0.0 | 0.0 ± 0.0 |
| 10 | 100.0 ± 0.0 | 100.0 ± 0.0 | 10 | 0.0 ± 0.0 | 0.0 ± 0.0 |
| 20 | 100.0 ± 0.0 | 100.0 ± 0.0 | 20 | 40.0 ± 0.18 | 5.0 ± 0.510 |
| 40 | 100.0 ± 0.0 | 100.0 ± 0.0 | 40 | 60.0 ± 10.0 | 40.0 ± 12.0 |
| 60 | 100.0 ± 0.0 | 100.0 ± 0.0 | 60 | 85.0 ± 10.0 | 45.0 ± 14.0 |
| 80 | 100.0 ± 0.0 | 90.0 ± 6.2 | 80 | 40.0 ± 0.13 | 10.0 ± 0.6 |

*Values represent percentages ± standard error

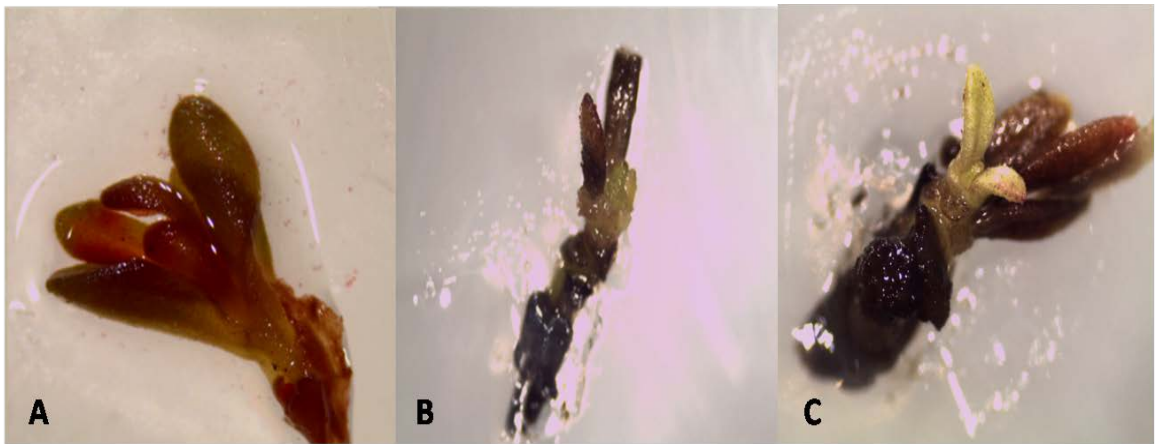


**Figure 2:** Survival and regrowth of vitrified and cryopreserved shoot tips of *Thymbra spicata* L. var. *spicata* after 60 min loading with the loading solution before exposure to PVS2 and LN. A: Survival using TTC test, B: Start of regrowth after 3 weeks, C: Regrowth after 8 weeks

*3.1.3. Effect of Loading Solution Type*

The survival and regrowth rates varied with loading solution type in both non cryopreserved and cryopreserved shoot tips of *Thymbra spicata* L. var. *spicata*. The highest survival and regrowth rates were obtained in non-cryopreserved shoot tips loaded with 2 M glycerol + 0.4 M sucrose (Table 4). Also, the highest survival and regrowth rates in the cryopreserved shoot tips of *Thymbra spicata var. spicata* were (60%) and(10%), respectively, were recorded only in those treated with 2 M glycerol + 0.4 M sucrose (Table 4). On the other hand, a significant decline in survival rate and/or no survival were recorded by tips treated with the rest types (Table 4). Using 2 M glycerol + 0.4 M sucrose as a loading solution was reported as best combination in many plant species, such as wild crocus (Baghdadi *et al*., 2011), sweet potato (Hirari and Sakai, 2003), potato (Kaczamrczyk *et al*., 2011), thyme (Marcco-Medina *et al.*, 2012), grapevine (Markovic *et al.*, 2013), African violet (Mogs *et al.*, 2003), *Artemisia herba alba* (Sharaf *et al.*, 2012), citrus (Sakai *et al.*, 1991) and potato (Zhao *et al.*, 2005) and many others which made it the most popular loading solution described in vitrification protocols (Sakai and Engelmann, 2007). This was attributed to the ability of glycerol to penetrate the cell membrane and yet to reduce the amount of intracellular water unlike the components in the other types which penetration is restricted on cell wall only (Tao and Li, 1986).

Moreover, using 10% DMSO + 0.75 M sucrose combination for cryoprotection had a negative effect on *Thymbra. spicata* L. var. *spicata* shoot tips (Table 4) which might be due to the high toxicity of this combination that yielded a damaging effect on plant cells even before exposure to PVS2 and LN. Meanwhile, the low survival and regrowth rates of cryopreserved shoot tips of *T. spicata* L. var. *spicata* might refer to the short exposure duration (only 20 min) to the loading solution and/or inability of these combinations to increase cell resistance to PVS2 toxicity and cryogenic freezing (Subaih *et al*., 2007; Kaviani, 2011).

*3.1.4. Effect of Plant Vitrification Solution (PVS) Type*

In the fourth vitrification experiment, survival and regrowth, rates varied according to the type of verification solution PVS used in the three different experiments (Table 4). In the first experiment when (2 M glycerol + 0.4 M sucrose) was used as a loading solution before exposure to LN, the result showed a significant decrease in regrowth (60%) in the non-cryopreserved shoot tips after exposure to PVS3 compared to the other treatments (Table 5).

Markovic *et al.* (2013) reported similar results as the highest recovery rate (50%) was obtained in grapevine exposed to PVS2 compared to complete death resulted in PVS3 treated shoot tips, which was due to the high toxicity of PVS3.

Moreover, after cryopreservation only shoot tips exposed to either PVS2 or (30% DMSO + 1 M sucrose) were able to survive and recover (Table 5) and the maximum survival (75%) and regrowth (35%) rates were recorded in shoot tips exposed to PVS2 before cryopreservation while no s urvival nor regrowth were obtained in shoot tips treated with (15% DMSO + 1 M sucrose) or PVS3 (Table 4). Meanwhile, the results obtained when (15% DMSO + 1 M sucrose) was used as a vitrification solution were very low (Table 5). This could be due to the fact that this vitrification solution was not a good option as it failed to fulfill proper dehydration requirements needed to prevent ice crystallization and cryogenic injury (Sakai and Engelmann, 2007).

**Table 4:** Survival and regrowth percentages of vitrified shoot tips of *Thymbra spicata* L. var. *spicata* as affected by different loading solutions

| Non- cryopreserved (-LN) | | | Cryopreserved (+LN) | | |
|---|---|---|---|---|---|
| Loading solution type | Survival % | Regrowth % | Loading solution type | Survival % | Regrowth % |
| 1M sucrose | 100.0 ± 0.0* | 65.0 ± 6.10 | 1M sucrose | 0.0 ± 0.0 | 0.0 ± 0.0 |
| 2M glycerol + 0.4M sucrose | 100.0 ± 0.0 | 100.0 ± 0.0 | 2M glycerol + 0.4M sucrose | 60.0 ± 6.1 | 10.00 ± 2.53 |
| 5% DMSO + 0.25M sucrose | 95.0 ± 5.0 | 85.0 ± 10. 0 | 5% DMSO + 0.25M sucrose | 0.0 ± 0.0 | 0.0 ± 0.0 |
| 5% DMSO + 0.5M sucrose | 100.0 ± 0.0 | 95.0 ± 5.0 | 5% DMSO + 0.5M sucrose | 20.0 ± 6.3 | 0.0 ± 0.0 |
| 10% DMSO + 0.25M sucrose | 90.0 ± 6.1 | 70.0 ± 3.51 | 10% DMSO + 0.25M sucrose | 20.0 ± 5.0 | 0.0 ± 0.0 |
| 10% DMSO + 0.5 M sucrose | 70.0 ± 9.0 | 55.0 ± 9.3 | 10% DMSO + 0.5 M sucrose | 0.0 ± 0.0 | 0.0 ± 0.0 |

*Values represent percentages ± standard error

In the second treatment when (10% DMSO + 0.5 M sucrose) was used as a loading solution for *Thymbra spicata* L. var. *spicata* shoot tips, PVS2 and (30% DMSO + 1 M sucrose) yielded the higher recovery rates after cryopreservation than those obtained when (10% DMSO + 0.75 M sucrose) was used as a loading solution, as only few shoot tips exposed to either PVS2 or (30% DMSO + 1 M sucrose) survived after cryopreservation (Table 5). This might be a result of double failure of both loading and PVS solutions to minimize the hazards of chemical toxicity that yielded a massive death of shoot tips after cryopreservation (Shatnawi *et al.*, 2011) (Table 5). Moreover, in all experiments data showed that using (2 M glycerol + 0.4 M sucrose) as a loading solution yielded the best results (Table 5).

This was in full agreement with many related research studies in which this loading solution was described as the best formula to be used before exposure to vitrification solution such as results obtained in sweet potato (Hirai and Sakai 2003), thyme (Marcco-Medina *et al.*, 2012), grapevine (Markovic *et al.*, 2013), citrus (Sakai *et al.*, 1991) and potato (Zhao *et al.*, 2005) which made this formula the most widely used in vitrification protocols (Sakai and Engelmann 2007).

However, in a co mplete contrast to the results obtained in *Thymbra spicata* L. var. *spicata*, Baghdadi *et al*. (2011) reported (15% DMSO + 1 M sucrose) as the best PVS solution for cryopreservation of wild crocus that might lead to a conclusion that the optimum PVS solution needed for successful cryopreservation is species-dependent.

**Table 5:** Survival and regrowth percentages of vitrified shoot tips of *Thymbra spicata* L. var. *spicata* as affected by vitrification solution type

| Loading solution type | Vitrification solution type | Survival % | Regrowth % |
|---|---|---|---|
| Non- cryopreserved (-LN) | | | |
| 2M glycerol+ 0.4M Sucrose | PVS2 | 100.0 ± 0.0* | 100.0 ± 0.0 |
| | 30%DMSO+1M sucrose | 100.0 ± 0.0 | 100.0 ± 0.0 |
| | 15%DMSO+1M sucrose | 100.0 ± 0.0 | 100.0 ± 0.0 |
| | PVS3 | 90.0 ± 6.2 | 60.0 ± 12.7 |
| Cryopreserved (+LN) | | | |
| 2M glycerol+ 0.4M Sucrose | PVS2 | 75.0 ± 11.1 | 35.00± 3.3 |
| | 30%DMSO+1M sucrose | 50.0 ± 8.2 | 15.00± 1.0 |
| | 15%DMSO+1M sucrose | 0.0 ± 0.0 | 0.0 ± 0.0 |
| | PVS3 | 0.0 ± 0.0 | 0.0 ± 0.0 |
| Non- cryopreserved (-LN) | | | |
| 10% DMSO+ 0.5M Sucrose | PVS2 | 100.0 ± 0.0 | 100.0 ± 0.0 |
| | 30%DMSO+1M sucrose | 100.0 ± 0.0 | 100.0 ± 0.0 |
| | 15%DMSO+1M sucrose | 100.0 ± 0.0 | 100.0 ± 0.0 |
| | PVS3 | 95.0 ± 5.0 | 75.0 ± 11.1 |
| Cryopreserved (+LN) | | | |
| 10% DMSO+ 0.5M Sucrose | PVS2 | 45.0 ± 4.0 | 15. 0 ± 2.07 |
| | 30%DMSO+1M sucrose | 30.0 ± 7.1 | 5.0 ± 1.9 |
| | 15%DMSO+1M sucrose | 0.0 ± 0.0 | 0.0 ± 0.0 |
| | PVS3 | 0.0 ± 0.0 | 0.0 ± 0.0 |
| Non- cryopreserved (-LN) | | | |
| 10% DMSO+ 0.75M Sucrose | PVS2 | 100.0 ± 0.0 | 100.0 ± 0.0 |
| | 30%DMSO+1M sucrose | 100.0 ± 0.0 | 100.0 ± 0.0 |
| | 15%DMSO+1M sucrose | 100.0 ± 0.0 | 100.0 ± 0.0 |
| | PVS3 | 100.0 ± 0.0 | 90.0 ± 6.1 |
| Cryopreserved (+LN) | | | |
| 10% DMSO+ 0.75M Sucrose | PVS2 | 25.0 ± 1.8 | 0.0 ± 0.0 |
| | 30%DMSO+1M sucrose | 10.0 ± 0.61 | 0.0 ± 0.0 |
| | 15%DMSO+1M sucrose | 0.0 ± 0.0 | 0.0 ± 0.0 |
| | PVS3 | 0.0 ± 0.0 | 0.0 ± 0.0 |

*Values represent percentages ± standard error

*3.2. In vitro Conservation Using Droplet Vitrification Technique*

The results showed that, in none- cryopreserved shoot tips, a full survival and regrowth rates were recorded in all treatments (Table 6) which could be attributed to the application of PVS2 at 0 °C that optimized the absorption rate of the solution by the cells, and yet eliminated the hazard of chemical toxicity and excessive dehydration during treatment with the PVS2. S imilar results were obtained by Matsumoto *et al*. (1998) as they reported very high levels of regrowth in wasabi shoot tips loaded with PVS2 solution at 0°C for up to 60 min without exposure to LN.

Meanwhile, after cryopreservation, survival and regrowth rates of *Thymbra spicata* L. var. *spicata* shoot tips varied with PVS2 exposure duration. The highest survival and regrowth rates were recorded when PVS2 exposure duration was (45 min) as (80% and 35%) survival and regrowth rates were recorded, respectively (Table 6, Figure 3). On the other hand, decreasing PVS2 exposure duration resulted in a decline in both the survival and the regrowth rates or even complete death after cryopresevation (Table 6).

These results agree with Markovic *et al*. (2013), who succeeded in conserving grapevine shoot tips using droplet vitrification technique, where (50 min exposure to PVS2 at 0°C) yielded the highest regrowth rate (Markovic *et al*., 2013). Similarly, a longer exposure period to PVS2 was needed to obtain higher regrowth rates in cryopreserved shoot tips of *Diospyros kaki* where exposure duration of 120 min at 0°C improved significantly the recovery rate (Niu *et al.*, 2010). However, in another related study, cryopreserved wasabi shoot tips had the highest regeneration rate after only (30 min) exposure to full strength PVS2 at 0°C (Matsumoto *et al.*, 1998).

So it can be concluded that the exposure duration to the PVS2 is considered critical for maximizing level of shoot tip recovery after vitrification and cryopreservation and largely affected by the plant species (Sakai and Engelmann 2007). Also, the use of aluminum strips could make it faster and easier to transfer of shoot tips into and out of LN which was described as highly important during the treatment with PVS2 as a slight elongation in exposure durations could be toxic for shoot tips (Kaczmarczyk *et al.*, 2011).

**Table 6:** Survival and regrowth percentages of vitrified shoot tips of *Thymbra spicata* L. var. *spicata* as affected by different exposure durations to plant vitrification solution PVS2 using droplet vitrification technique

| Non-cryopreserved (-LN) | | | Cryopreserved (+LN) | | |
|---|---|---|---|---|---|
| Duration (min.) | Survival % | Regrowth % | Duration (min.) | Survival % | Regrowth % |
| 0.0 | 100.0 ± 0.0* | 100.0 ± 0.0 | 0.0 | 0.0 ± 0.0 | 0.0 ± 0.0 |
| 10 | 100.0 ± 0.0 | 100.0 ± 0.0 | 10 | 0.0 ± 0.0 | 0.0 ± 0.0 |
| 20 | 100.0 ± 0.0 | 100.0 ± 0.0 | 20 | 40.0 ± 10. 0 | 0.0 ± 0.0 |
| 30 | 100.0 ± 0.0 | 100.0 ± 0.0 | 30 | 55.0 ± 9.0 | 10.0 ± 0.61 |
| 45 | 100.0 ± 0.0 | 100.0 ± 0.0 | 45 | 80.0 ± 12.2 | 35.0 ± 1.5 |

*Values represent percentages ± standard error

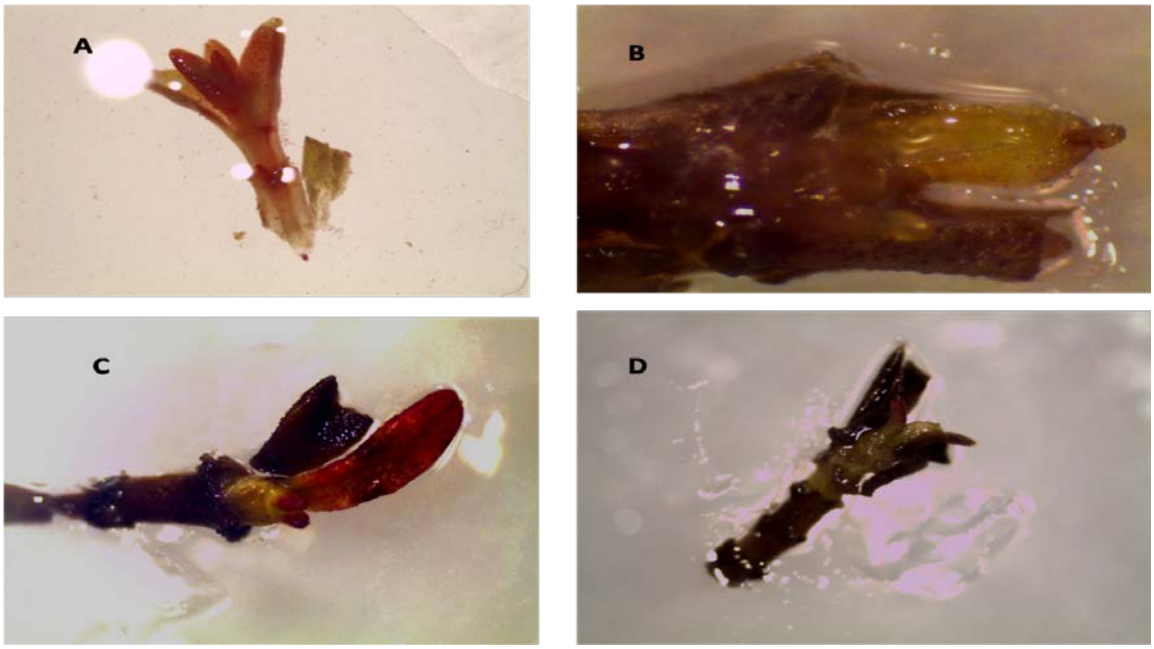


**Figure 3.** Survival and regrowth of vitrified and cryopreserved shoot tips of *Thymbra spicata* L. var. *spicata* after 45 min exposure to PVS2 at 0 ºC before exposure to LN using droplet vitrification technique. A: Survival using TTC test, B: Start of regrowth after 3 weeks. C: Regrowth after 5 weeks, D: Regrowth after 8 weeks

### *3.3. Assessment Genetic Stability Using Amplified Fragment Length Polymorphism (AFLP) Technique*

No differences between *Thymbra spicata* L. var. *spicata* shoot tips were obtained before and after exposure to liquid nitrogen (Figure 4). Similar results were obtained in other plant species, such as strawberry (Caswell and Kartha, 2009), cork oak (Fernandez *et al.*, 2008), pea (Keller *et al.*, 2006) and *Vanda pumila* (*Orchidaceae*) (Na and Kondo, 1996). Cryopreservation is speculated to maintain the stored material genetically stable and although it might result in cellular injury, there is no clear connection of this injury to any genetic alteration of the stored plants (Kaczmarczyk *et al*., 2012). Furthermore, Harding (2004) reported that no genetic differences were confirmed at morphological, histological and molecular levels in most cryopreserved samples of many species, including Prunus, sugarcane, onion, kiwi, Eucalyptus, coffee, Dendrobium and Cosmos and if any changes happened, this could be attributed to other factors, such as tissue culture, cryoprotection and regeneration process but not only to the cryogenic treatment itself.

Moreover, the results showed genetic differences between the seed-originated cryopreserved shoot tips of *Thymbra spicata* L. var. *spicata* and the wild mother plant sample and another seed-originated sibling tissue cultured plant (Figure 4). The genetic differences between the seed-originated cryopreserved shoot tips of *Thymbra spicata* L. var. *spicata* and the wild mother plant were expected and reflecting the nature of cross pollination existing of this plant in nature (Akine *et al*., 2010).

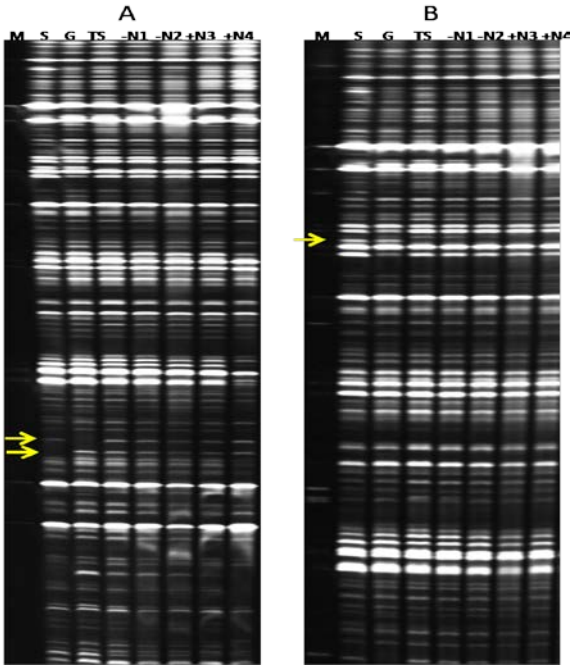


**Figure 4.** DNA banding patterns using AFLP for *Thymbra spicata* L. var. *spicata*. Pre-amplified DNA products were selectively amplified using two *Eco*RI+*MseI* primer combinations: A= E-ACT IR Dye 700 with M-CTA, B= E-ACT IR Dye 700 with M-CTC. M= DNA Marker, S= Wild mother plant, G= Green house plant, TS= Tissue cultured plant, -N1 = Before cryopreservation, -N2= Before cryopreservation, +N3= After cryopreservation, +N4= After cryopreservation

As a conclusion, endangered *Thymbra spicata* L. var. *spicata* was successfully introduced to cryogenic conservation using vitrification, droplet vitrification and encapsulation- vitrification techniques. Also, cryopreservation proved its ability to maintain genetic fidelity of the stored plants, as AFLP analysis showed no difference between the shoot tips before and after exposure to LN. However, more research on measures to improve recovery rates after cryopreservation is needed, such as preculture treatments, optimizing PVS2 and loading solution exposure duration before exposure to LN, in addition to post thawing and recovery treatments.

## Acknowledgment

Authors would like to thank Hamdi Mango Center for Scientific Research (HMCSR) at The University of Jordan, the Royal Botanic Garden, RBG (Tell Al- Rumman) and the National Center for Agricultural Research and Extension NCARE , for their support and help.

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JJBS
Jordan Journal of Biological Sciences

Volume 10, Number 1,March 2017
ISSN 1995-6673
Pages 29 - 32

# Evaluation of the Toxicological Effects of *Senecio aureus* Extract on the Liver and Hematological Parameters in Wistar Rats

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Received: November 25, 2016 Revised: January 24, 2017 Accepted: January 29, 2017

**Abstract**

The use of *Senecio aureus* for therapeutic purposes is well known; however, in recent times, much concern has been raised regarding the toxicity of this plant extract used in treatment of gynecological disorders and jaundice. The sub-chronic oral toxicity of *Senecio aureus* was investigated. 16 male wistar rats were divided into four groups: control and treatments (100, 300 and 500 mg/kg body weight). Each group received the graded dose of the extract by gavage daily for 28 days while control received water. The administration of the extract resulted in significantly ($p<0.05$) increased total bilirubin and cholesterol and level from 29.14±1.18mg/dl and 83.00±7.16 mg/dl in the control to 53.19±6.11mg/dl and 166.67±14.03mg/dl, respectively, at dose of 500 mg/Kg body weight. In the hematological analysis, there were no significant changes compared to the control group ($p<0.05$). The present result suggests that the prolonged use of this plant extract at high dose in the treatment of diseases could be detrimental to the liver.

**Keywords**: : *Senecio auerus*, toxicity, jaundice, liver, hematology.

## 1. Introduction

The use of herbal remedies for the treatment of diseases has a long history and medicinal plants and their derivatives are still used in different parts of the world in one form or the other (Akhtar et al., 2009; Ekor, 2014). Herbal remedies have also been incorporated and consumed as food because of their acclaimed therapeutic effects and believe that the active principle in these herbs work together synergistically (Perumal-Samy and Gopalakrishnakone, 2010; Kasilo and Trapsida, 2011; Ekor, 2014; Si-Yuan, et al., 2014). The advocates of the consumption of herbals often claim that plant drugs are relatively non-toxic, safe and even free from any serious side effects (Goyal et al., 2007; Akhtar et al., 2009; Kasilo and Trapsida, 2011). However, scientific evaluation of plants often show that irrational consumption of these medicinal plants for their active principle have become one of the major causes hepatotoxicity and mortality around the globe (Gurib-Fakim, 2005; Tagliati et al., 2008; Akhtar et al., 2009; Kalatari and Rastmantsh, 2009).

Although a number of herbals are used in treating liver diseases, reports are accumulating about liver injury after intake of herbals including those advertised for the treatment of liver diseases (Stickel and Schuppan, 2007; Wurochekke et al., 2008). Acute and/or chronic liver damage have been reported after ingestion of some Chinese herbs and herbs that contain pyrolizidine alkaloids, kava, atractylis gummifera, senna alkaloids (Haller et al., 2002). Like many synthetic drugs, herbals undergo metabolic activation to form reactive metabolites often associated with drug toxicity (Zhang et al., 2015). The magnitude of the effect depends on the inherent toxicity of the substance, its route and duration of exposure to a particular organism, (Kenneth, 2000). Therefore, safety continues to be a major issue with the use of herbal remedies, hence, it becomes necessary to put in place appropriate measures to ensure that all herbal medicines are safe and of suitable quality.

The plant *senecio aureus* has a long historical use in some local communities of Adamawa State, Nigeria for the treatment of assorted gynecological disorders, speeding up protracted labor and relief of labor pains. It is also used in the treatment of excessive vaginal discharge as well as several kinds of menstrual problems. The plant is also used in the treatment of jaundice in children and infants. A previous study of the plant revealed that the plant was effective against $CCl_4$ induced liver damage, in the acute toxicity study single doses of up to 4000mg/Kg body weight was safe and resulted in dose dependent increase in Packed Cell Volume (PCV).

There is a high degree of concern regarding the safe use of this plant as its variant from other parts of the world has been reported to be medicinal while others reported it as toxic even at low doses (Wojcikowski et al., 2004).

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However, variability in active ingredients due to environmental/growing conditions is known to affect most herbs; for this reason, the present study evaluated the toxicological impact of the plant extracts on the liver and hematological parameters in animal model.

## 2. Materials and methods

### *2.1. Collection of Plants Materials*

Fresh *senecio aureus* plants were collected around footpaths by the football field and guest house of Modibbo Adama University of Technology Yola. The plant was authenticated in the Department of Plant Science, Modibbo Adama University of Technology, Yola. They were then air-dried and pulverized to fine powder.

### *2.2. Animals for the Experiment*

Wistar rats (80-100g) were obtained from the National Veterinary Research Institute (NVRI), Vom Plateau State and were allowed to acclimatize for fifteen days prior to the commencement of the experiment. The animals were housed under standard experimental conditions and fed food and water ad libitum, vital feeds from Grand cereals and oil mills Jos were used.

### *2.3. Preparation of Plant Extract*

Five hundred gram (500g) of the pulverized plant material was suspended in 2500 ml of 70% ethanol and allowed for 48 hours after which the mixture was sieved using a cheese cloth and then filtered using Whatman No-1 filter paper. The filtrate was concentrated to dryness in a water bath at 50°C and the yield was calculated as a percentage of the starting material.

### *2.4. Sub-Chronic Toxicity Study of the Plant Extract*

The sub-chronic toxicity study was carried out according to the method described by Hassan et al. (2008). Four groups of rats were used in the study and each group consisted of four rats. The dried extract was dissolved in distilled water as vehicle of administration. The group and treatment were as follows:

Group I: Control group treated with distilled water.

Group II: *Senecio aureus* 100 mg/kg body weight of extract per day.

Group III: *Senecio aureus* 300 mg/kg body weight of extract per day.

Group IV: *Senecio aureus* 500 mg/kg body weight of extract per day.

Animals were fed food and water ad libitum. Each group received the respective treatment orally by gavage, once a day for 28 days and was observed for signs of toxicity.

### *2.5. Collection of Serum and Blood Samples*

After the 28 days of treatment, the rats were fasted overnight and anesthetized using chloroform. Animals were sacrificed by cardiac puncture and blood samples collected. Blood samples were collected in EDTA bottle for hematological analysis and plain bottles for biochemical analysis. Samples for biochemical analysis were centrifuged at 2500 rpm for 10 minutes. The sera obtained were used for biochemical analysis.

### *2.6. Analysis of Biochemical Indices of Liver Function*

Evaluation of liver function, activities of Alanine Aminotransferase (ALT), Aspartate Aminotransferase (AST), Alkaline Phosphatase (ALP), triglycerides, cholesterol, bilirubin, albumin and total protein in sera were determined using Randox diagnostic kits.

### *2.7. Hematological Studies*

Hematological analyses were performed using automatic hematology analyzer (Coulter USA) on the whole as described by the manufacturer.

### *2.8. Statistical Analysis*

Results are expressed as Mean ± SEM. Statistical analysis was performed by one way Analysis of Variance (ANOVA) followed by Tukey multiple comparison tests using graph pad prism 5. p values < 0.05 were considered significant.

## 3. Results and Discusson

*Senecio aureus* is utilized locally by some communities and indigenous healers to treat liver diseases especially jaundice as well as gynecological disorders. The safety of *Senecio aureus* on the liver was evaluated by determining its effects on enzyme and non-enzyme markers of the liver as well as hematological parameters. The results of this experiment showed that groups treated with *Senecio aureus* and control showed no visible changes in behavior and signs of intoxication during the 28-day period. However, at the end of the experiments, the percentage increase in weight of the animals receiving 300 and 500 mg/kg body weight of the extract when compared to the control were significantly ($p<0.05$) different as shown in Table 1.

**Table 1:** Showing the initial average weight, final average weight and percentage weight increase at the end of the experiment

| Treatment | Control | 100 mg/kg | 300 mg/kg | 500 mg/kg |
|---|---|---|---|---|
| Initial weight (g) | 106.5±6.05 | 108.68±7.95 | 116.98±9.90 | 119.90±7.03 |
| Final weight (g) | 189.78±8.32 | 172.65±6.43 | 170.00±10.53 | 161.60±8.43 |
| Weight increase (%) | 78.19±7.81 | 58.86±5.91 | 45.32±9.00* | 34.78±7.03* |

Values are Mean ± SEM, n=4. Values with * significantly decreased relative to the control ($p<0.05$).

The assay of liver enzymes is an important index in liver diagnosis, disease investigation and assessment of drugs or plant extracts for safety/toxicity risk (Hassan et al., 2008).The greater the degree of damage in the liver, the higher the activities of these enzymes in the serum (Cheesbrough, 1991). In the present study, the administration of *Senecio aureus* extract for 28 days resulted in increased levels of AST and ALT, the increased levels of AST was not significant ($p<0.05$) as to suggest liver damage. This is very useful as more people are utilizing this plant as a traditional medicine today than they were a few years ago. Although some herbal medicines have a promising potential, many of them remain untested and their uses are usually not monitored.

This makes knowledge of their potential adverse effects very limited (Ekor, 2014). Levels of marker enzymes of liver injury increased significantly whenever the liver is compromised (Trung et al., 2010). The results of the enzyme and non-enzyme markers of the liver function after 28 days of treatment are shown in Tables 2 and 3. The results show that there was a dose-dependent increase in ALT, AST and ALP but these increases were not significant ($p<0.05$) compared to the control group as to suggest any damage to the liver. This dose-dependent increase suggests that a dose greater than 500 mg/kg for prolong periods, *Senecio aureus* may not be safe for the treatment of the diseases. The ALP activities generally increased during the experiment (Table 2) but not significant ($p<0.05$) as to suggest any damage. Increased ALP may be due to intra hepatic obstruction of the bile flow (Nwachukwu and Iweala, 2009).

**Table 2**: Results of post treatment of rats with ethanolic extract of *Senecio aureus* on some enzyme markers of liver function

| Groups | AST(U/L) | ALT(U/L) | ALP(U/L) |
|---|---|---|---|
| Control | 35.00±2.92 | 12.25±1.44 | 50.00±3.00 |
| 100 mg/kg | 42.75±4.52 | 14.00±2.12 | 47.22±4.70 |
| 300 mg/kg | 44.50±6.59 | 16.00±1.96 | 57.64±4.99 |
| 500 mg/kg | 49.00±6.68 | 18.25±0.96 | 61.11±5.07 |

Values are Mean ± SEM, n=4. No significant changes were observed compared to the control group ($p<0.05$)

The decreasing serum albumin concentration resulting from the administration of the extract as the dose of the extract increase (Table 3) suggests that high doses of these extracts may impair the absorption of protein in the intestine or even liver damage (Grant and Kachmar, 1987; Ozaki et al., 1991). Bilirubin is mainly formed form the breakdown of hemoglobin in the cells of the liver, spleen and bone marrow. As the liver becomes irritated, the total bilirubin becomes elevated which may be a result of liver cell damage or bile duct damage within the liver itself (Ochei and Kolhatkar, 2005). In the present study there was a significant ($p>0.05$) increase in total bilirubin at dose of 500mg/kg indicating possible damage to the liver.

The cholesterol and triglyceride levels increased in the entire treated group but the increase in triglyceride was not significant ($p<0.05$) compared to the control group but the increase in cholesterol was significant compared to the control at 500 mg/Kg. The liver has a major role in controlling the plasma level of LDL cholesterol; it synthesizes cholesterol, removes cholesterol from lipoprotein remnant. Increased level of serum cholesterol may be a result of the impaired liver function (Vasudevan and Sreekumari, 2007).

**Table 3**: Results of non-enzyme markers of liver function after 28 days of administration of extract of *Senecio aureus*

| Groups | C.B (µmol/L) | T.B (µmol/L) | Albumin (mg/dl) | T.G (mg/dl) | Cholesterol (mg/dl) |
|---|---|---|---|---|---|
| Control | 25.22± 3.68 | 29.14± 4.43 | 29.46± 1.18 | 85.40± 13.15 | 83.00± 7.16 |
| 100 mg/kg | 25.83± 2.56 | 35.08± 1.20 | 28.26± 1.95 | 102.08± 13.77 | 131.25± 26.65 |
| 300 mg/kg | 28.91± 4.20 | 46.25± 5.75 | 28.53± 0.96 | 106.25± 15.73 | 125.00± 11.28 |
| 500 mg/kg | 35.06± 1.85 | 53.19± 6.11* | 25.45± 1.12 | 116.67± 9.00 | 166.67± 14.03* |

Values are Mean ± SEM; n=4, values with * significantly increased relative to the control ($p<0.05$), C.B: Conjugated bilirubin, T.B: Total bilirubin, and T.G: Triglycerides

Although the PCV of the *Senecio aureus* treated groups increased as the dose increased but this increase was not significantly different from the control ($p<0.05$). The present study, therefore, does not reveal significant effects on hematological parameters (Table 4), suggesting that the extract may not have had any effect on the blood production system of the body. The results of the present study suggest that there could be hepatotoxicity of the biliary cirrhosis when the extract is taken for a longer period, at higher dose.

**Table 4**: Results of hematological parameters of treated and control animals after 28 days of treatment with ethanolic *Senecio aureus* extract

| Groups | PCV % | T. WBC Cells/mm$^3$ | Neutrophils % | Monocytes % | Lymphocytes % |
|---|---|---|---|---|---|
| Control | 41.50± 3.48 | 23333± 6467 | 18.33± 4.41 | 3.00± 1.53 | 75.33± 2.60 |
| 100 mg/kg | 39.75± 1.38 | 18466± 3525 | 15.00± 2.89 | 2.33± 1.77 | 82.67± 2.19 |
| 300 mg/kg | 43.00± 1.08 | 23000± 9800 | 25.00± 5.00 | Not detected | 75.00± 5.00 |
| 500 mg/kg | 48.00± 1.08 | 20700± 2506 | 21.25± 3.15 | 2.75± 1.31 | 76.00± 3.94 |

Values are Mean ± SEM, n=4. Basophils and Eosinophils were not detected in any of the samples during differential count. None of the hematological parameters showed any significant difference relative to the control ($p<0.05$)

## 4. Conclusion

From the results of the present study, the use of *Senecio aureus* for the treatment diseases at high dose and prolonged periods will have an adverse effect on the liver. Therefore, the persistent use of the plant in the treatment of the diseases should be discouraged.

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JJBS
Jordan Journal of Biological Sciences

Volume 10, Number 1,March 2017
ISSN 1995-6673
Pages 33 - 36

# Molecular Identification and Evolutionary Relationship of the New Record *Callistethus sp.7VF-2014* (Coleoptera:Scarabaeidae:Reutelinae) in North of Iraq

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*Received: June 3, 2016* *Revised: November 27, 2016* *Accepted: December 18, 2016*

**Abstract**

A total of 15 specimens of the *Callistethus sp.7VF-2014* were collected from different parts in Duhok Governorate during period extended from May to July 2015. The present study was done for molecular identification and evolutionary relationships according to DNA sequence and RNA secondary structure of *Callistethus sp.7VF-2014*. The partial nuclear 28S ribosomal (rDNA) sequence (365 bps) of this species was amplified with universal primers and sequenced by monitoring genetic analyzer. Molecular identification and phylogenetic relationships were carried out using 28S rDNA region. The outcome demonstrated that the query sequence was more than 98% identical to this species of the insect. The phylogenetic tree revealed 96- 98% relationships of the sequence of *Callistethus sp.7VF-2014* and 28S rDNA regions for other Gen Bank stored species of *Callistethus*. Likewise, the present evolutionary finding was affirmed by the molecular morphometrics, according to the secondary structure of 28S rDNA region. The topology investigation created same information as the gained tree. In conclusion, DNA sequence analysis recorded the species *Callistethus sp.7VF-2014* for the first time in Duhok City, Kurdistan region, Iraq. Phylogenetic evolutionary as indicated by both; DNA primary sequence and RNA secondary structure can be considered as a valuable tools for separating species of *Callistethus*.

**Keywords:** *Callistethus sp.7VF-2014*, 28S rDNA, molecular identification, phylogeny, molecular morphometric.

## 1. Introduction

The genus *Callistethus* was proposed by Blanchard (1851). Potts (1974) considered *Callistethus* as a synonym of *Anomala* when working on Neotropical Anomalini. Morón (1997) listed six Mexican species of Anomalini as members of *Callistethus*. However, in phylogenetic studies in view of species of 18 genera of Anomalini from around the globe supported the monophyly of *Callistethus* permitting another recognizable proof for it (Ramírez *et al.*, 2009). The monophyly of the genus *Callistethus* and its definition in relation to the genus *Anomala* had been widely questioned, but no consensus has been reached (Jameson *et al*., 2003).

Based on the checklist by Krajcik (2007), the genus *Callistethus* comprises 142 species distributed in Asia (85 species) and America (57 species), although their real diversity is probably much higher, included only one species from Nepal, *C. consularis* Blanchard, 1851. Since then, a few other Asiatic species have been described in this genus, such as *C. stoliczkae* Sharp, 1878, *C. seminitidus* Fairmaire, 1889, *C. pterygophorus* Ohaus, 1903, and *C. umidicauda* Arrow, 1912. Ramírez-Ponce and Morón, (2012) described a new species *Callistethus tlapanecus*, in eastern mountains of the Guerrero, Mexico.

Based on molecular data, only broader studies not focused on Anomalini (García *et al*., 2013 and Ahrens *et al*., 2014). Uses of molecular markers in the phylogenetic studies of various organisms have become increasingly important in recent times. Although widely practiced even now, traditional morphology based systems of classification of organisms have some limitations. On the other hand, it appears that the use of molecular markers, though relatively recent in popularity and are not free entirely of flaws, can complement the traditional morphology based method for phylogenetic studies (Patwardhan *et al*., 2014). Filippini *et al.* (2015) carried out another phylogenetic analysis of *Callistethus* and *Anomala* species, based on sequences of one nuclear (a

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fragment of 28S) and two mitochondrial (16S and COI) genes, described 11 new species from the revision of the genus *C. allistethus, C. carbo*, *C. flavodorsalis*, *C. fuscorubens, C. lativittis, C. levigatus*, *C. macroxantholeus*, *C. microxantholeus*, *C. multiplicatus*, *C. parapulcher*, *C. pseudocollaris* and *C. stannibractea*, and also propose a new synonym, new combinations, from Costa Rica.

28S rRNA gene sequence for many major metazoan groups has become available in the recent years. Also, efforts to align sequences according to the secondary structure model for 28S rRNA of these organisms have become valuable for the purpose of phylogenetic evolutionary analyses (Manzari *et al*., 2002 and Schmidt *et al*., 2006).

*Callistethus sp.7VF-2014*, recently identified as a new species, was described in National Center for Biotechnology Information (NCBI) (*https://www.ncbi.nlm.nih.gov/Taxonomy/Browser /wwwtax.cgi?lvl=0&id=1447034*) and Universal Protein Resource (UniProt) (*http://www. uniprot. org/taxonomy/1447034*). In the present study, the first objective is to confirm molecularly the presence of *Callistethus sp.7VF-2014* in Kurdistan region, Iraq. This goes more specifically to describe the molecular identification of *Callistethus sp.7VF-2014* using 28S rDNA sequence. The study was also set out with the aim of performing evolutionary development with other species of the *Callistethus* and to do the secondary structure modeling as a support of the primary molecular finding.

## 2. Material and Methods

### *2.1. Study Area and Sample Collection*

Fifteen specimens of the *Callistethus sp.7VF-2014* collected from different parts in Duhok Governorate by light traps from Zawita 19/5 /2014, Bamarne 22/5 /2014, Sarsank 15/6 /2014, Swartka 21/6/2014 and Sarke 25/6/2014


**Figure 1.** Map of Iraq showing Duhok city and the red color points showing exact location of samples.

### *2.2. Extraction, Amplification and Sequencing of DNA*

The genomic DNA of *Callistethus sp.7VF-2014* samples was obtained by employing AccuPrep® Genomic DNA Extraction Kit (BIONEER, KOREA) according to the manufacture's instruction with few modifications (incubation time of tissue lyses step was extended into 3 hours and utilized absolute ethanol instead of isopropanol for DNA precipitation). The samples were macerated in mortar and pestle, and the contents were transferred into sterile tube contain 200 µl tissue lysis buffer and kept in incubator at 60 ˚C for 3 hrs. Qualification and quantification of DNA concentration was determined by using NanoDrop (ND- 1000, USA).

The targeted region of partial 28S rDNA was amplified by PCR using universal primers, forward primer C1 (5′-ACC CGC TGA ATT TAA GCA T-3′ at position 25), and the reverse primer C3 (5′-CTC TTC AGA GTA CTT TTC AAC-3′ at position 390), they were designed and selected by (Mollaret *et al*., 2000). A total of 50 µl volume of reaction mixture was prepared to contain 2 µl DNA template, 25 µl OnePCR$^{TM}$ master mix (GENEDIREX, KOREA), 1 µl for each primers and 21 µl double deionized water (dd$H_2O$) in a thermocycler MJ Research, Applied Biosystem (AB). The cycling profile consisted of an initial denaturation step of 5 min at 94˚C followed by 35 cycles at 94˚C, 45 sec. at 51˚C, 45 sec. at 72˚C 45 sec., and final extension 5 min at 72˚C. The PCR products were run in 2% agarose gel electrophoresis. Bands stained with SYBR green were visualized on an UV transilluminator using 100 bp ladder (GENEDIREX, KOREA), expected size of the PCR amplicon was 365 bps.

DNA sequencing, using both forward and reverse primers was performed separately by ABI 3130X genetic analyzer (SINGAPORE). The PCR fragments of the *Callistethus sp.7VF-2014* were excised from the agarose gel and used as a source of DNA template for sequence specific PCR amplification.

### *2.3. Sequence and Structure-Based Phylogeny*

Muscle multiple sequence alignment program (http://www.drive5.com/muscle/) was employed to compare consensus sequences with the neglectful gap and expansion penalties. The results of 28S rDNA regions were entered in the MEGA 6.0 program (http://en.bio-soft.net/tree/MEGA.html) for constructing the evolutionary developmental trees. The phylogenetic tree of the *Callistethus sp.7VF-2014* was constructed using character state method (maximum likelihood). Branch support was given, employing 1000 bootstrap replicates. Secondary structures of 28S rDNA regions of *Callistethus sp.7VF-2014* were expected by the online MFold package (version 3.5) (*http://unafold.rna.albany.edu/?q=mfold*). MFold is the broad employed algorithms for secondary structure of RNA expectation that are dependent on a search for the minimal free energy state.

## 3. Results

### *3.1. Molecular Identification*

The expected amplicon size for 28S rDNA of *Callistethus sp.7VF-2014* is supposed to be 365 bp (Figure 2), but the results obtained from sequencing were only 349 bp (missing 19 bp due to the quality of amplified sequence). BLAST program from Gen bank (*http://blast.ncbi.nlm.nih.gov/*) was used to compare our amplified sequences with other stored species of *Callistethus* sequences. The results got from the BLAST indicated that the query sequence was more than 98% identical to *Callistethus sp.7VF-2014* (Figure 3).

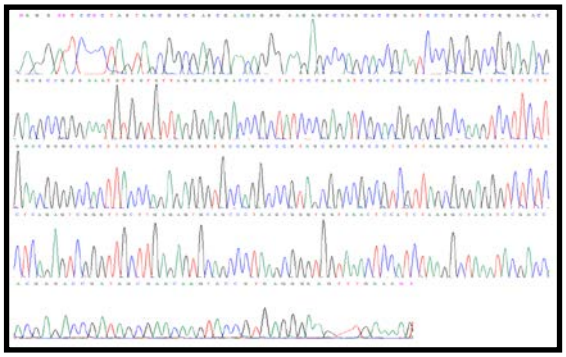

**Figure 2.** The chromatography sequence result of 28S rDNA sequence of *Callistethus sp.7VF-2014*

```
Query  1    AACTAACTAGGATTCCCCTAGTAGCGGCGAGCGAACAGGGAAGAGCCCAGCACCGAATCC  60
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  20   AACTAACTAGGATTCCCCTAGTAGCGGCGAGCGAACAGGGAATAGCCCAGCACCGAATCC  79

Query  61   CGCGGCCGGTTGACGGACGCCGGGAAATGTGGTGTTAGGGAGGACCCGCTATCCCGAGAT  120
            ||||||||||||||||||||||||||||||||||||||||||||| |||| ||| ||||||
Sbjct  80   CGCGGCCGGTTGACGGACGCCGGGAAATGTGGTGTTAGGGAGGATCCGCCATCTCGAGAT  139

Query  121  CGCACGGCGCGCCCAAGTCCTTCTTGAACGGGGCCACTTACCCAGAGAGGGTGCCAGGCC  180
            || ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  140  CGTTCGGCGCGCCCAAGTCCTTCTTGAACGGGGCCACTTACCCATAGAGGGTGCCAGGCC  199

Query  181  CGTAGTTGGCCGCCGTCGTTAGCGGGAGGGTCTCTCCTCAGAGTCGGGTTGCTTGAGAGT  240
            |||||||||||||||||| ||||||||||||||||||||||||||||||||||||||||||
Sbjct  200  CGTAGTTGGCCGCCGTCGATCGCGGGAGGATCACTCCTCAGAGTCGGGTTGCTTGAGAGT  259

Query  241  GCAGCCCTAAGCGGGTGGTAAACTCCATCTAAGGCTAAATACGACCACGAGACCGATAGC  300
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  260  GCAGCCCTAAGCGGGTGGTAAACTCCATCTAAGGCTAAATACGACCACGAGACCGATAGC  319

Query  301  GAACAAGTACCGTGAGGGAAGTTTGAAAAG  330
            |||||||||||||||||| ||||||||
Sbjct  320  GAACAAGTACCGTGAGGGAAAGTTGAAAAG  349
```

**Figure 3.** Pairwise alignment of 28S rDNA sequence of *Callistethus sp.7VF-2014*. Query is the study or sample sequence and Sbjct is the GenBank sequence

### *3.2. DNA Sequence Based Phylogeny*

Phylogenetic tree according to primary sequence showed 96-98% relationships in comparing the sequence of *Callistethus sp.7VF-2014* and stored 28S rDNA regions for the other species of *Callistethus*. Phylogenetic analysis employing the diverse method, maximum likelihood method as mentioned in Figure 4. It was revealed and indicated that the topology was the same among acquired trees with significant bootstrap support for the clades. For the bootstrap analysis, the values of 70% and above represented the accuracy of evolutionary development and showed reliable grouping among various species of *Callistethus*. The tree topologies according to phylogenetic analysis showed *Callistethus sp.7VF-2014* as molecularly closely related with *Callistethus sp*. 6VF-2014 and *Anomala sp*- 206 species (Figure 4).

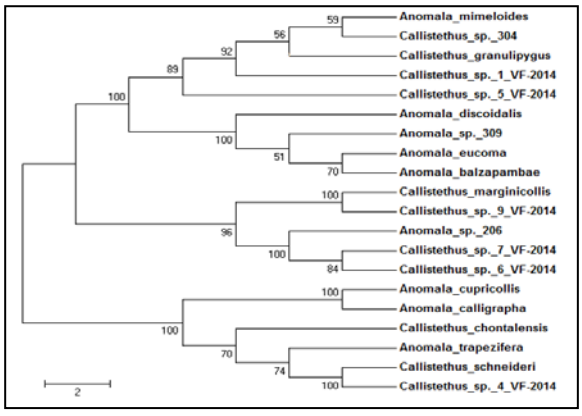


**Figure 4.** Phylogenetic positioning of *Callistethus sp.7VF-2014* according to sequences of 28S rDNA employing maximum likelihood method, (Tamura 3-parameter model with invariant sites)

### *3.3. RNA Structure-Based Phylogeny*

Predict 28S rDNA secondary structural properties with the highest negative free energy ΔG= -131.60 kcal/mol of *Callistethus sp.7VF-2014* to provide the principal information for evolutionary developmental analysis (Figure 5). The secondary sequence properties of 28S rDNA structure, as represented in the figure, were analyzed according to loops and conserved stems. In the structure of *Callistethus sp.7VF-2014* the loops arrangements exhibit according to their numbers and classified into: interior loop, hairpin loop, bulge loop, multi loop and exterior loop (Table 1). The topology was only based on the expected RNA secondary structure of 28S rDNA region which determined most associations among the species researched.

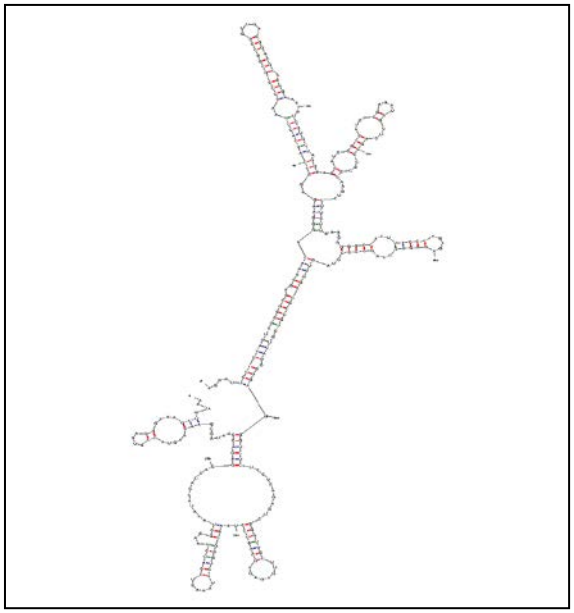

**Figure 5.** Schematic representation of the 28S rRNA expected secondary sequence of *Callistethus sp.7VF-2014*

**Table 1.** Arrangement of various loops of 28S rDNA sequence region of *Callistethus sp.7VF-2014*

| Type of loops | Number of loops |
|---|---|
| Interior | 8 |
| Hairpin | 6 |
| Bulge | 5 |
| Multi | 3 |
| Exterior | 1 |

## 4. Discussion

*Callistethus sp.7VF-2014* species genetically distinguished from the other available species of *Callistethus* previously identified with the same rDNA region marker, available at the GenBank in National Center for Biotechnology Information (NCBI). The primary sequence analysis using universal primers of study sample revealed that the insect belongs to species *Callistethus sp.7VF-2014* (Figure 3).

Phylogeny is the history of descent of a group of taxa such as species from their common ancestors including the order of branching and sometimes the times of divergence.

In many cases, evolution of nucleotide sequences is more or less affected by various kinds of constraints. In order to compensate for such factors and to estimate precise relationships among taxi, a l arge variety of phylogenetic technique has been devised (Simon *et al*., 1994 and Mindell *et al.*, 1996). For phylogenetic inference, the obtained data sequences were analyzed, using Maximum Likelihood method (ML) analysis.

Dendrogram in (Figure 4) shows how closely these species are related to each other's. The aim of producing a dendrogram is to visualize the best relationships of the phylogenetic (Evolutionary history) between the studied species *Callistethus sp.7VF-2014* and the other 19 species which belong to the same tribe: Anomalini, subfamily Reutelinae and family: Scarabaeidae. *Callistethus sp.7VF-2014*, *Callistethus sp.*6VF-2014 and *Anomala sp*. 206 species form monophyletic group, because they are descended from a s ingle common ancestor. This result agrees with that of Ramírez *et al.* (2009) who supported the monophyly of the *Callistethus* species. Jameson *et al*. (2003) also supported monophyly of the genus *Callistethus* and genus *Anomala*. In another study, Filippini *et al*. (2015) recorded that the *Callisthethus* is subordinate in *Anomala* and thus most likely *Anomala* has either to be split in several genera or *Callisthethus* to be included in Anomala with increasing phylogenetic knowledge.

Furthermore, phylogenetic discoveries were affirmed utilizing secondary structure investigation as a tool of computational biology, RNA loops were used for amendment the alignment. Molecular morphometrics is expected to analyze phylogenetic relationships depend on similarities among some structural proprieties of folded nucleotide molecules (Bernard *et al.*, 2000). Molecular morphometrics has been suggested to be the most strengthened tool in comparison to classical primary structure analysis, due to the only consideration of the size variations for homologous structural segments, whereas molecular morphometrics representing the folding pattern of RNA molecule (Bernard *et al.*, 2000). The topology of the present study, based on t he expected RNA secondary sequence of the 28S rDNA structures, revealed and identified most associations among the species studied; for instance the structure of *Callistethus sp.7VF-2014*, total number loops preference were 23 loops, types of the loops according to their numbers were exterior loop (1), multi loop (3), bulge loop (5), hairpin loop (6) and interior loop (8), respectively as presented in Figure 5 a nd Table 1. Incorporation of secondary structure information allows improved estimates of phylogeny among several *Callistethus* species.

In conclusion, DNA sequencing of the sample study revealed the first recording of *Callistethus sp.7VF-2014* in Duhok City, Kurdistan region, Iraq. Phylogenetic evolutionary as indicated by both, DNA primary sequence and RNA secondary structure can be considered as valuable tools for separating species of *Callistethus*.

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JJBS
Jordan Journal of Biological Sciences

Volume 10, Number 1,March 2017
ISSN 1995-6673
Pages 37 - 38

# *Morinda lucida* Benth. S (Rubiaceae)- New record from India

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*Received: December 17, 2016 Revised: January 24, 2017 Accepted: January 29, 2017*

**Abstract**

*Morinda lucida* Benth. (Rubiaceae) a N igerian species has not been reported so far from India. This species has been collected for the first time from coastal regions of Tamil Nadu. A detailed description of phenology, ecology with illustrations of the species is provided.

**Keywords:** *Morinda lucida* Benth., Rubiaceae, New record, Coastal region, India.

## 1. Introduction

The genus Morinda Benth belonging to the family Rubiaceae Juss. The genus consists of 90 s pecies (Mabberley, 2008). In India, the genus is represented by c. 8 species (Hooker, 1988), and in Tamil Nadu by c. 5 species (Henry et al., 1983). During the floristic exploration in the coastal regions from Tranquebar, Nagapattinam district, Tamil Nadu an interesting species of Morinda Benth was collected, after critical studies and consultation with specimens deposited at MH (Botanical Survey of India) Coimbatore. It was identified as *Morinda lucida* Benth. (Figs. 1 & 2) and, therefore, it is an addition to Indian flora. *Morinda lucida* Benth., occurs only from Senegal to Sudan and southward to Angola and Zambia, Nigeria. It has neither been collected nor reported from India (Henry, 1987; Gamble, 1957; Hooker, 1872; Matthew, 1987). The voucher specimen has been deposited in the Bharathiar University Herbarium (Bharathi) Coimbatore, Tamil Nadu, India.


Figure 1: Natural Habit of *Morinda lucida* Benth. S **A**. Stem; **B**. Lower surface of leaf; **C.** Flower; **D**. & **E**. Fruit

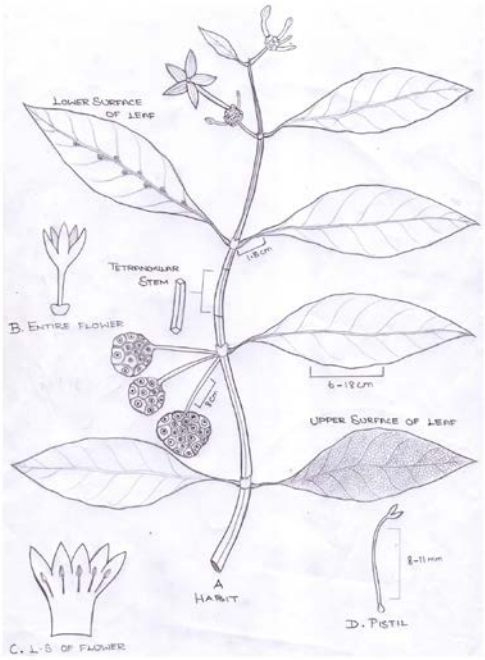


**Figure 2**: Illustration of *Morinda lucida* Benth. S

## 2. Affinities

Morinda lucida Benth. is morphologically similar to M. citrifolia and Morinda coreia Buch.-Ham. Morinda coreia Buch.-Ham, chiefly differs from the former in having chartaceous seed not winged.

## 3. Key to the Species

1a.Seeds winged……2
b.Seeds not winged…….....1.*M.coreia*

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2a. Leaves glabrous, shining; fruit yellow...2.M.citrifolia
b. Leaves slightly hairy, without shining; fruit brown……..…….3.*M.lucida*

**Description**

Shrub evergreen or small to medium-sized tree up to 18(–25) m tall, with bole and branches often crooked or gnarled; bark smooth to roughly scaly, grey to brown, often with some distinct purple layers. Leaves opposite, simple and entire; stipules ovate or triangular, 1–7 mm long, falling early; petiole up to 1.5 cm long; blade elliptical, 6–18 cm × 2–9 cm, base rounded to cuneate, apex acute to acuminate, shiny above, sometimes finely pubescent when young, later only tufts of hairs in vein axils beneath and some hairs on the midrib. Inflorescence a stalked head 4–7 mm in diameter, 1–3 at the nodes opposite a single leaf; peduncle up to 8 cm long bearing at base a stalked cup-shaped gland. Flowers bisexual, regular, 5-merous, heterostylous, fragrant; calyx cup-shaped, c. 2 mm long, persistent; corolla salver-shaped, c. 1.5 cm long, white or greenish yellow, lobes ovate-lanceolate, up to 5 mm × 2 .5 mm; ovary inferior, 2-celled, style 8–11 mm long with 2 stigma lobes 4–7 mm long; stamens 5, inserted in the corolla throat, with short filaments. Fruit a drupe, several together arranged into an almost globose succulent syncarp 1–2.5 cm in diameter, soft and black when mature; pyrene compressed ovoid, up to 6.5 mm × 4 mm, dark red-brown, very hard, 1-seeded. Seed ellipsoid, c. 3.5 mm × 2 mm × 0.5 mm, yellowish, soft.

**Flowering & Fruiting**: October – January**.**

**Uses:** The fruits are edible, used to treat diabetes**.**

**Habitat:** Coastal regions near 25 m, species associated with *M. coreia*.

**Distribution**: Tropical Africa, Ethiopia and India.

Specimen examined: India: Tamil Nadu, Coastal regions from Tranquebar, Nagapattinam district, October 30th 2016.V. Dhaarani, Field number: 306 (Bharathi).

## 4. Conclusion

This appreciated plant was not cited previously in any India floras and the species is found growing abundantly in its new habitat in Tamil Nadu state and therefore, it is likely that it may soon become naturalized and may occupy larger areas in the near future.

## Acknowledgement

We are grateful to the Professor and Head, Department of Botany, Bharathiar University, Coimbatore, Tamil Nadu, India for providing the necessary facilities.

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JJBS
Jordan Journal of Biological Sciences

Volume 10, Number 1,March 2017
ISSN 1995-6673
Pages 39 - 48

# Vitamin E and/or Wheat Germ Oil Supplementation Ameliorate Oxidative Stress Induced by Cadmium Chloride in Pregnant Rats and Their Fetuses

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Received: August 1, 2016 Revised: January 24, 2017 Accepted: February 5, 2017

**Abstract**

The present study aims to investigate the protective effect of Vit. E and/or WGO against Cd induced toxicity in pregnant rats and their fetuses. Thirty pregnant rats were divided into five groups; Control, CdCl2, CdCl2+Vit. E, CdCl2+WGO and CdCl2+Vit. E+WGO. Oral administration of CdCl2 caused impairment in the hematological parameters as indicated by significant ($P<0.05$) decrease in RBCs, Hb, Hct, PLt s and WBCs in maternal rats and their fetuses. CdCl2 administration caused disturbances in the hepatic and renal functions as reflected by significant ($P<0.05$) increase in ALT, AST, creatinine and urea. Also, CdCl2 administration caused an oxidative stress in the liver and kidney tissues of maternal rats and their fetuses. In addition, CdCl2 induced growth retardations as observed by significant ($P<0.05$) depletion in fetal body weight, length and the number of alive fetuses and significant ($P<0.05$) increase in the number of dead, absorbed and malformed fetuses. The pronounced abnormalities were: exencephaly, exophthalmia, open eyelids, microtia, short tail, short fore and hind limbs, umbilical hernia, and curvature in the vertebral column as compared to control group. The presence of Vit. E and/or WGO with CdCl2 improved the all examined parameters. These natural substances could exhibit a protective effect in preventing physiological alterations and fetal malformations due to their potent antioxidant properties.

**Keywords:** Cadmium, vitamin E, wheat germ oil, pregnant rats, fetuses.

## 1. Introduction

Cadmium (Cd) is one of the major occupational and environmental pollutants. Human exposure to Cd occurs chiefly through inhalation or ingestion. Cadmium is considerably toxic with destructive impacts on most organ systems such as respiratory, digestive, reproductive, skeletal and cardiovascular systems and some sensitive organs, including liver and kidney (Jama *et al*., 2013). In addition, Cd induced malformations of the neural tube, craniofacial region, limbs, trunk, viscera, and axial skeleton in fetuses when administered during gestation (EL-Sayed *et al.*, 2013). Cd acts as a stimulator for formation of Reactive Oxygen Species (ROS), hydrogen peroxide also, hydroxyl radicals. These free radicals, enhance lipid peroxidation, DNA damage, altered calcium and sulfhydryl homeostasis (Sevcikova *et al*., 2011).

Antioxidants are substances that protect cells against the adverse effects of xenobiotics, toxicants, drugs and carcinogens. These antioxidants, such as vitamin C, vitamin E, omega-3 fatty acid and wheat germ oil, can be supplemented through diet and have been utilized in a prophylactic manner against toxic substances induced oxidative stress (Aboubakr *et al*., 2014).

Vitamin E (Vit. E) is a fat-soluble antioxidant. It plays an important role in retarding the pathogenesis of different decadence diseases; cancer, inflammatory diseases, neurological disorders and chronic vascular diseases through its function of inhibiting free radical-mediated tissue damage. It is essential for the development of early embryos during implantation as well as for the protection of the fetus against oxidative damage (Wilkinson *et al.*, 2005; Traber & Manor, 2012).

Wheat Germ Oil (WGO) is unique among dietary supplements. It contains some B complex vitamins ($B_6$, $B_{12}$ and folic acid) that are essential in the formation of red blood cells. It was claimed to be anti-inflammatory and described as a suitable natural antioxidant due to its high content of Vit. E. WGO acts as a p rotection against oxidative stress appeared to be mediated through decreasing the pro-oxidants and enhancement of cellular antioxidant activities. WGO is rich in unsaturated fatty acids, mainly oleic, α-linoleic and functional phytochemicals mainly flavonoids, sterols, octacosanols and glutathione (Alessandri *et al.*, 2006). It also has a

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number of nutritional and health useful, improving physical fitness and probably retarding effects of aging (Megahed, 2011).

The present work is designed to evaluate the possible protective effects of vitamin E and/or wheat germ oil against cadmium chloride – induced toxicity in pregnant rats and their fetuses.

## 2. Materials And Methods

### *2.1. Chemicals*

Cadmium chloride anhydrous (98%-Cd pure white powder, 100 g package), vitamin E (α- tocopherol acetate) and wheat germ oil were obtained from Kahira Pharma and Chem. Ind. Co. (Cairo–Egypt) in the form of soft, gelatinous capsules.

### *2.2. Experimental Animals*

Sexually mature female albino rats weighing from 200-210 g were obtained from Faculty of Medicine, Alexandria University, Alexandria, Egypt. Rats were kept on basal diet and tap water *ad libitum*. They were acclimated under controlled environmental conditions at room temperature (25±2ºC) with humidity (50±10%) and a 12h light/dark cycle. For mating purposes, four females were housed overnight with two males starting at 21:00 h. Females were checked by 7:00 h the next morning, and the presence of a vaginal plug was designated as gestational day zero. The experiments and the protocol were carried out according to the guidelines of the National Institutes of Health (NIH).

### *2.3. Animal Groups*

Thirty pregnant rats were divided randomly into five groups, six per each group as follows:

**Control group:** Each pregnant rat was orally received distilled water and 0.5 ml corn oil as a vehicle.

**Cadmium chloride–treated group:** Each pregnant rat was orally received cadmium chloride at a dose 5 mg/kg BW/day (1/20 $LD_{50}$) (ATSDR, 2008).

**Cadmium chloride+vitamin E–treated group:** Each pregnant rat was orally received cadmium chloride at a dose 5 mg/kg BW/day and intraperitoneally (IP) with vitamin E at a dose 100 mg/kg BW/day (Mahabady and Varzi, 2011).

**Cadmium chloride+wheat germ oil–treated group:** Each pregnant rat was orally received cadmium chloride at a dose 5 mg/kg BW/day and wheat germ oil at a dose 54 mg/kg BW/day (Reddy *et al*., 2000).

**Cadmium chloride+vitamin E+wheat germ oil-treated group:** Each pregnant rat was orally received cadmium chloride at a dose 5 mg/kg BW/day and intraperitoneally (IP) with vitamin E at a dose 100 mg/kg BW/day and orally with wheat germ oil at a dose 54 mg/kg BW/day.

All groups were treated with different treatments for 13 days from 6$^{th}$ to 18$^{th}$ day of gestation.

### *2.4. Maternal and Fetal Endpoints*

All of the pregnant rats were sacrificed by ether anesthesia at the 19$^{th}$ day of gestation and fetuses were removed from the uterus. The implantation sites, corpora lutea, living, dead and reabsorbed fetuses were counted. Live fetuses were weighed, and photographed by HD digital camera (Samsung 10x) for evaluating externally visible abnormalities, according to the technique of Wilson's (1978).

### *2.5. Examination of the Fetus Gross Morphology and the Skeleton*

After a brief autopsy one and half of the fetuses were fixed in 10% formalin, examined under stereomicroscope for the occurrence of any malformation. Fetuses were examined for skeletal malformation through two procedures for skeletal staining according to the method of McLeod (1980).

### *2.6. Collection and Preparation of Blood Samples*

Blood samples were collected from anesthetized mother and their fetuses into sterile tubes. The first part of blood was collected in tubes containing EDTA for determination of hematological parameters (RBCs, Hb, Hct, platelet counts and WBCs). The second part allowed to clot and centrifuged at 3000 rpm for 20 min. Serum was separated and stored at -20°C for determination of some biochemical parameters.

### *2.7. Biochemical Parameters*

Determination of serum aspartate aminotransferase (AST; EC 2.6.1.1) and alanine aminotransferase (ALT; EC 2.6.1.2) were estimated using kits from Sentinel Ch. (Via principle Eiagen 5-20155 kit, Milano, Italy) according to Reitman and Frankel (1957) method. Serum creatinine and urea were estimated by using the methods of Bowers and Wong (1980) and Fawcett and Scott (1960), respectively. Liver and kidney MDA were measured as Thiobarbituric Acid Reactive Substance (TBARS) (Ohkawa *et al*., 1979). Also, the levels of reduced GSH (Beulter *et al*., 1963) and the antioxidant enzyme activities, including the catalase (CAT; EC 1.11.1.6) (Aebi, 1984), superoxide dismutase (SOD; EC 1.15.1.1) (Nishikimi *et al.*, 1972) and glutathione peroxidase (GPx; EC 1.1.1.9) (Chiu *et al*., 1976) were assayed.

### *2.8. Statistical Analysis*

The results were analyzed using the SPSS computer software package version 19.0 (Chicago, IL, USA). Data were presented as mean±SE. Data were evaluated by one-way ANOVA followed by Least Significant Difference (LSD). Values were considered statistically significant at $P<0.05$.

## 3. Results

### *3.1. Effect of Cdcl2, Vit. E, WGO and/or Their Combinations on the Values of RBCs, Hb, Hct, PLts and WBCs in Maternal and Their Fetuses*

$CdCl_2$–maternal treated rats and their fetuses showed significant ($P<0.05$) decrease in the values of RBCs, Hb, Hct, PLts and WBCs as compared to control. While, the administration of Vit. E and/or WGO with $CdCl_2$ showed a significant increase ($P<0.05$) in the measured hematological parameters as compared to $CdCl_2$–maternal treated rats and their fetuses (Table 1).

**Table 1**: Effect of $CdCl_2$, Vit. E, WGO and/or their combination, on the values of RBCs, Hb, Hct, PLt s and WBCs in maternal and their fetuses

| Parameters / Groups | RBCs ($\times10^6$cell/µl) | | Hb (g/dl) | | Hct (%) | | PLt ($\times10^3$ cell /µl) | | WBCs ($\times10^3$cell/µl) | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Mother | Fetus | Mother | Fetus | Mother | Fetus | Mother | Fetus | Mother | Fetus |
| Control | 4.52 ±0.26 | 4.60 ±0.16 | 13.70 ±0.22 | 13.54 ±0.62 | 45.33 ±1.53 | 44.80 ±2.86 | 283.40 ±26.42 | 408.60 ±10.95 | 8.72 ±1.02 | 13.98 ±3.56 |
| $CdCl_2$ | 2.74 ±0.63$^{a}$ | 1.54 ±0.11$^{a}$ | 6.44 ±0.66$^{a}$ | 4.34 ±0.27$^{a}$ | 23.60 ±4.98$^{a}$ | 16.80 ±2.17$^{a}$ | 222.80 ±16.38$^{a}$ | 167.40 ±22.14$^{a}$ | 4.60 ±1.14$^{a}$ | 5.20 ±0.87$^{a}$ |
| $CdCl_2$+Vit. E | 4.02 ±0.37$^{bc}$ | 4.10 ±0.25$^{bc}$ | 11.44 ±0.81$^{ab}$ | 11.72 ±0.39$^{abc}$ | 38.80 ±3.56$^{ab}$ | 35.80 ±2.59$^{abc}$ | 300.40 ±17.11$^{bc}$ | 262.20 ±22.11$^{ab}$ | 6.96 ±1.29$^{ab}$ | 6.50 ±1.32$^{ab}$ |
| $CdCl_2$+WGO | 3.28 ±0.13$^{ab}$ | 3.14 ±0.21$^{ab}$ | 9.86 ±0.23$^{ab}$ | 9.44 ±0.65$^{ab}$ | 30.80 ±1.92$^{ab}$ | 25.40 ±3.65$^{ab}$ | 288.20 ±13.55$^{b}$ | 185.80 ±23.33$^{ab}$ | 10.36 ±1.03$^{abc}$ | 6.94 ±0.22$^{ab}$ |
| $CdCl_2$+Vit.E+WGO | 4.40 ±0.25$^{bc}$ | 3.99 ±0.36$^{ab}$ | 12.50 ±0.91$^{bc}$ | 11.30 ±0.63$^{ab}$ | 40.40 ±4.34$^{bc}$ | 35.20 ±3.35$^{ab}$ | 303.40 ±17.97$^{bc}$ | 337.00 ±27.12$^{abc}$ | 8.55 ±0.62$^{ab}$ | 8.68 ±1.11$^{abc}$ |

Values are expressed as mean ±S.E., n=6 for each group. Mean values within column not sharing common superscript letters (a, b, c) were significantly different ($P< 0.05$)

### *3.2. Effect of CdCl2, Vit. E, WGO and/or Their Combinations on the Serum Activities of AST and ALT in Maternal and Their Fetuses*

Results presented in Table 2 showed a significant increase in the ALT and AST serum activities, in cadmium-maternal treated group and their fetuses when compared with the control group. While, administration of Vit. E and/or W GO with $CdCl_2$ showed significant ($P<0.05$) d ecrease in the activities of AST and ALT as compared to $CdCl_2$-maternal treated rats and their fetuses.

**Table 2**: Effect of $CdCl_2$, Vit. E, WGO and/or their combination on the serum activities of AST and ALT in maternal and their fetuses

| Parameters / Groups | AST (U/L) | | ALT (U/L) | |
|---|---|---|---|---|
| | Mother | Fetus | Mother | Fetus |
| Control | 62.92±1.89 | 43.83±3.54 | 41.58±4.45 | 37.50±3.27 |
| $CdCl_2$ | 378.85±22.11$^{a}$ | 72.00±1.90$^{a}$ | 286.33±10.05$^{a}$ | 78.50±2.59$^{a}$ |
| $CdCl_2$+ Vit. E | 200.03±5.80$^{ab}$ | 50.08±2.13$^{ab}$ | 161.92±1.88$^{ab}$ | 38.87±0.77$^{bc}$ |
| $CdCl_2$+ WGO | 191.17±4.12$^{abc}$ | 47.08±4.96$^{ab}$ | 116.67±2.07$^{ab}$ | 63.00±3.80$^{ab}$ |
| $CdCl_2$+ Vit. E+ WGO | 100.38±4.33$^{ab}$ | 38.00±1.10$^{abc}$ | 57.92±6.33$^{abc}$ | 45.30±4.79$^{ab}$ |

Values are expressed as mean±S.E. n=6 for each group. Mean values within column not sharing common superscript letters (a, b, c) were significantly different ($P < 0.05$).

### *3.3. Effect of CdCl2, Vit. E, WGO and/or Their Combination on the Serum Contents of Urea and Creatinine in Maternal and Their Fetuses*

Table 3 indicates that the levels of serum urea and creatinine in the serum of maternal and their fetuses were significantly ($P<0.05$) increased in $CdCl_2$– treated group as compared to the control group. I n contrast, the administration of Vit.E and/or WGO with $CdCl_2$ caused a significant decline ($P<0.05$) in the serum concentrations of urea and creatinine in comparison with $CdCl_2$–maternal treated rats and their fetuses.

**Table 3**: Effect of $CdCl_2$, Vit. E, WGO and/or their combination on the serum concentrations of urea and creatinine in maternal and their fetuses

| Parameters / Groups | Urea (mg/dl) | | Creatinine (mg/dl) | |
|---|---|---|---|---|
| | Mother | Fetus | Mother | Fetus |
| Control | 47.07±3.04 | 46.71±4.45 | 0.37±0.15 | 0.42±0.074 |
| $CdCl_2$ | 78.67±5.47$^{a}$ | 65.50±2.81$^{a}$ | 0.91±0.12$^{a}$ | 0.93±0.08$^{a}$ |
| $CdCl_2$+ Vit. E | 54.67±2.07$^{ab}$ | 52.00±1.68$^{ab}$ | 0.63±0.18$^{ab}$ | 0.50±0.14$^{b}$ |
| $CdCl_2$+ WGO | 55.00±3.52$^{ab}$ | 50.00±0.64$^{ab}$ | 0.68±0.15$^{ab}$ | 0.70±0.14$^{ab}$ |
| $CdCl_2$+ Vit. E+ WGO | 41.72±4.21$^{bc}$ | 37.97±2.28$^{bc}$ | 0.33±0.16$^{bc}$ | 0.32±0.16$^{bc}$ |

Values are expressed as mean ±S.E. n =6 for each group. Mean values within column not sharing common superscript letters (a, b, c) were significantly different ($p <0.05$)

### *3.4. Effect of CdCl2, Vit. E, WGO and/or Their Combination on the Levels of Liver and Kidney MDA and GSH in Maternal and Their Fetuses*

The levels of MDA in the liver and kidney of maternal and their fetuses were significantly incremented ($P<0.05$) after administration of $CdCl_2$ as compared to the control group (Tables 4&5). While, the levels of hepatic and renal GSH were significantly declined ($P<0.05$) in $CdCl_2$–maternal treated group and their fetuses compared to control group. Meanwhile, the MDA and GSH levels in $CdCl_2$+Vit. E, $CdCl_2$+WGO and $CdCl_2$+Vit. E+WGO–treated groups were significantly improved in the liver and kidney tissues as compared to $CdCl_2$–maternal treated rats and their fetuses.

**Table 4**: Effect of $CdCl_2$, Vit. E, WGO and/or their combination on the levels of liver MDA and GSH in maternal and their fetuses

| Parameters / Groups | Liver | | | |
|---|---|---|---|---|
| | MDA (µmol/ g tissue) | | GSH (U/g tissue) | |
| | Mother | Fetus | Mother | Fetus |
| Control | 36.13±2.89 | 19.72±1.60 | 71.50±2.22 | 53.00±3.35 |
| $CdCl_2$ | 112.34±10.89$^{a}$ | 52.95±3.77$^{a}$ | 12.50±0.96$^{a}$ | 30.67±1.12$^{a}$ |
| $CdCl_2$+ Vit. E | 67.17±3.82$^{ab}$ | 30.00±0.64$^{ab}$ | 36.50±3.48$^{ab}$ | 57.00±1.90$^{bc}$ |
| $CdCl_2$+ WGO | 70.80±4.21$^{ab}$ | 30.33±2.80$^{ab}$ | 38.50±1.59$^{ab}$ | 42.00±1.90$^{ab}$ |
| $CdCl_2$+ Vit. E+ WGO | 48.00±0.64$^{abc}$ | 29.17±0.71$^{ab}$ | 72.50±1.59$^{bc}$ | 48.30±1.94$^{ab}$ |

Values are expressed as mean ±S.E. n=6 for each group. Mean values within column not sharing common superscript letters (a, b, c) were significantly different ($P < 0.05$)

**Table 5**: Effect of $CdCl_2$, Vit. E, WGO and/or their combination on the levels of kidney MDA and GSH in maternal and their fetuses

| Parameters / Groups | Kidney | | | |
|---|---|---|---|---|
| | MDA (µmol/g tissue) | | GSH (U/g tissue) | |
| | Mother | Fetus | Mother | Fetus |
| Control | 36.00±2.53 | 28.30±1.94 | 61.00±1.27 | 65.51±0.35 |
| $CdCl_2$ | 200.42±6.01$^{a}$ | 72.25±2.88$^{a}$ | 13.00±1.68$^{a}$ | 24.08±2.64$^{a}$ |
| $CdCl_2$+ Vit. E | 94.67±5.90$^{ab}$ | 49.00±1.27$^{ab}$ | 37.50±2.85$^{ab}$ | 44.50±2.85$^{ab}$ |
| $CdCl_2$+ WGO | 72.50±1.59$^{ab}$ | 37.67±3.01$^{ab}$ | 49.50±1.59$^{ab}$ | 50.75±1.14$^{ab}$ |
| $CdCl_2$+ Vit. E+ WGO | 51.00±1.90$^{abc}$ | 28.17±0.76$^{bc}$ | 59.00±3.16$^{bc}$ | 61.33±3.67$^{bc}$ |

Values are expressed as mean ±S.E. n =6 for each group. Mean values within column not sharing common superscript letters (a, b, c) were significantly different ($P < 0.05$)

### *3.5. Effect of CdCl2, Vit. E, WGO and/or Their Combination on the Activities of Liver and Kidney SOD, CAT and GPx in Maternal and Their Fetuses*

The activities of SOD, CAT and GPx in the liver and kidney of $CdCl_2$-maternal treated group and their fetuses were significantly declined ($P<0.05$) in comparison with the control group (Tables 6&7). Interestingly, the activities of SOD, CAT and GPx were significantly elevated ($P<0.05$) in $CdCl_2$+Vit. E, $CdCl_2$+WGO and $CdCl_2$+Vit. E+WGO–treated groups, when compared with the $CdCl_2$–maternally treated rats and their fetuses.

**Table 6**: Effect of $CdCl_2$, Vit. E, WGO and/or their combination on the activities of liver SOD, CAT and GPx in maternal and their fetuses.

| Parameters / Groups | Liver | | | | | |
|---|---|---|---|---|---|---|
| | SOD (U/mg protein) | | CAT (U/mg protein) | | GPx (U/mg protein) | |
| | Mother | Fetus | Mother | Fetus | Mother | Fetus |
| Control | 83.63±5.53 | 80.30±3.49 | 55.03±5.18 | 49.30±3.71 | 61.00±1.27 | 38.63±2.99 |
| $CdCl_2$ | 20.00±1.38$^{a}$ | 31.67±3.01$^{a}$ | 22.63±3.49$^{a}$ | 25.92±3.18$^{a}$ | 6.53±1.00$^{a}$ | 10.33±0.81$^{a}$ |
| $CdCl_2$+Vit. E | 49.08±1.72$^{ab}$ | 46.00±3.80$^{ab}$ | 50.25±2.30$^{bc}$ | 44.58±4.14$^{bc}$ | 27.20±2.17$^{ab}$ | 30.50±0.34$^{ab}$ |
| $CdCl_2$+WGO | 35.33±2.66$^{ab}$ | 40.00±2.53$^{ab}$ | 46.33±2.66$^{ab}$ | 35.00±2.53$^{ab}$ | 30.17±2.41$^{ab}$ | 33.50±3.48$^{ab}$ |
| $CdCl_2$+Vit.E+WGO | 65.50±2.85$^{abc}$ | 72.42±1.60$^{abc}$ | 58.87±3.90$^{bc}$ | 43.30±4.12$^{ab}$ | 49.50±3.42$^{abc}$ | 51.03±5.18$^{abc}$ |

Values are expressed as mean ±S.E. n =6 for each group. Mean values within column not sharing common superscript letters (a,b,c) were significantly different ($P<0.05$)

**Table 7**: Effect of $CdCl_2$, Vit. E, WGO and/or their combination on the activities of kidney SOD, CAT and GPx in maternal and their fetuses

| Parameters / Groups | Kidney | | | | | |
|---|---|---|---|---|---|---|
| | SOD (U/mg protein) | | CAT (U/mg protein) | | GPx (U/mg protein) | |
| | Mothers | Fetuses | Mothers | Fetuses | Mothers | Fetuses |
| Control | 88.63±4.83 | 49.00±2.53 | 52.80±2.54 | 47.53±4.58 | 40.80±1.69 | 48.63±2.23 |
| $CdCl_2$ | 17.30±1.94$^{a}$ | 22.83±3.56$^{a}$ | 29.28±5.38$^{a}$ | 39.83±2.45$^{a}$ | 6.50±0.96$^{a}$ | 7.50±0.34$^{a}$ |
| $CdCl_2$+ Vit. E | 49.00±1.42$^{ab}$ | 48.00±1.90$^{bc}$ | 42.00±4.15$^{ab}$ | 40.50±0.96$^{ab}$ | 38.08±1.89$^{b}$ | 46.33±2.96$^{bc}$ |
| $CdCl_2$+ WGO | 39.58±0.40$^{ab}$ | 39.50±0.34$^{ab}$ | 38.00±1.27$^{ab}$ | 44.42±4.14$^{b}$ | 35.32±3.12$^{ab}$ | 28.30±2.86$^{ab}$ |
| $CdCl_2$+Vit. E+WGO | 89.58±3.63$^{bc}$ | 50.00±3.80$^{bc}$ | 59.58±0.40$^{bc}$ | 50.67±1.60$^{bc}$ | 45.83±3.43$^{abc}$ | 44.50±2.85$^{bc}$ |

Values are expressed as mean ±S.E. n =6 for each group. Mean values within column not sharing common superscript letters (a, b, c) were significantly different ($P<0.05$)

### *3.6. Effect of CdCl2, Vit. E, WGO and/or Their Combinations on the Number and Size of Implanted Fetuses*

The uterus of the pregnant rats treated with $CdCl_2$ showed diminution in the number and size of implanted fetuses in addition to fetal absorption sites as compared to the uteri of control group (Figure 1: A & B, Table 8). In contrast, the uterus of the pregnant rats treated with Vit E and/or WGO showed the nearly normal appearance (Figure 1: C, D & E). The fetus body weight and length significantly ($P$<0.05) decreased compared to those of the control group. While, the body weight and length significantly increased ($P$< 0.05) in $CdCl_2$+Vit. E, $CdCl_2$ +WGO and $CdCl_2$+Vit.E+WGO-treated groups as compared to those of the fetus of $CdCl_2$- maternal treated rats (Table 8).

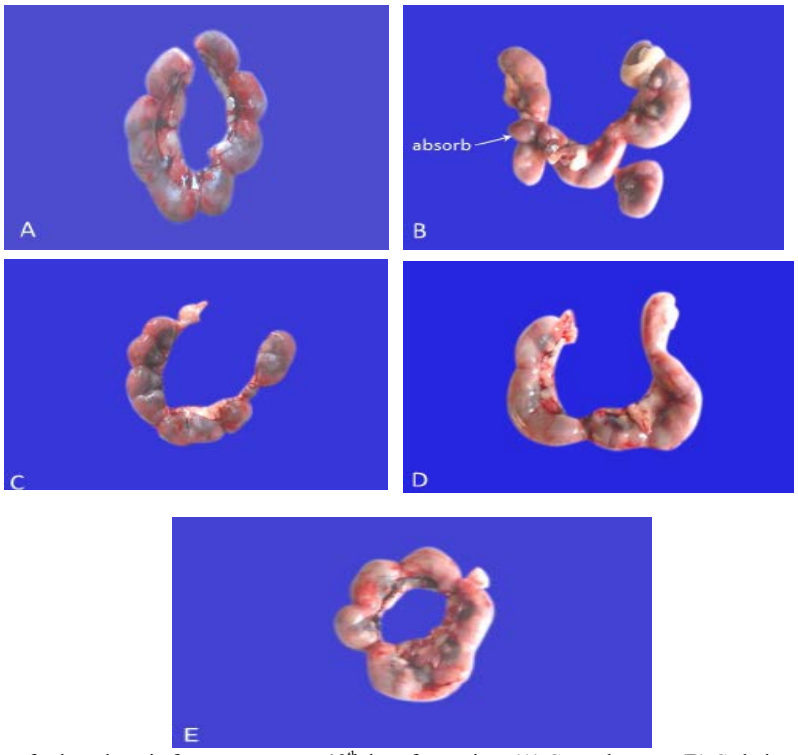



**Figure (1):** Photographs of selected uteri of pregnant rats on 19th day of gestation**: (A)** Control group. **(B)** Cadmium chloride $CdCl_2$ – treated group, showed uterine malformation and absorbed fetus (white arrow) **(C)** $CdCl_2$ + Vit.E - treated group. **(D)** $CdCl_2$+ WGO - treated group. **(E)** $CdCl_2$+Vit.E + WGO - treated group. Notice: C, D & E showed more or less normal appearance of the fetuses in the uterus

**Table 8**: Effect of $CdCl_2$, Vit. E, WGO and/or their combination on the number of alive, dead, absorbed, malformed, body weight and body length of fetuses on the 19th day of gestation

| Parameters / Groups | Fetuses on the 19th day of gestation | | | | | | |
|---|---|---|---|---|---|---|---|
| | No. of alive fetuses | No. of Dead fetuses | No. of absorbed fetuses | Malformed fetuses No. | % | Body weight (g) | Body length (Cm) |
| Control | 46 | - | - | - | - | 4.60±0.40 | 5.10±0.20 |
| $CdCl_2$ | 31 | 8 | 5 | 18 | 56. 25 | 2.20±0.40$^{a}$ | 3.10±0.20$^{a}$ |
| $CdCl_2$+Vit. E | 45 | - | - | - | - | 3.80±0.30$^{ab}$ | 3.90±0.30$^{ab}$ |
| $CdCl_2$+WGO | 43 | - | - | - | - | 4.40±0.50$^{abc}$ | 4.40±0.30$^{abc}$ |
| $CdCl_2$+Vit. E+WGO | 44 | - | - | - | - | 4.20±0.50$^{abc}$ | 4.90±0.50$^{abc}$ |

Values are expressed as mean ±S.E. n =6 for each group. Mean values within column not sharing common superscript letters (a, b, c) were significantly different ($P$<0.05)

### *3.7. Effect of CdCl2, Vit. E, WGO and/or Their Combinations on the Morphological Characters of Fetuses*

On the 19th day of gestation, the normal fetus slightly appeared with straight body in both back and neck regions (Figure 2: A). The head appeared straight and small in size in relation to the whole body. The eyes were closed with upper and lower eyelids and the external auditory canal was completely invisible, being covered by the well developed ear pinna. In those fetuses, different regions of both fore and hind limbs appeared with well-developed structures and their extremities showed the distinctive number of digits with clear demarcated phalanges. The abdominal region of normal fetuses displayed a cylindrical shape ending with the tail (Figure 2: A).

While, the external malformations in fetuses maternally treated with cadmium chloride were observed in figures 2 (B & B1) 3 (A & B) and 4 (A, B, C & D). The pronounced abnormalities were: exencephaly, exophthalmia, open eyelids, microtia, short tail, short fore and hind limbs, umbilical hernia, and curvature in the vertebral column as compared to control group. In contrast, administration of Vit. E, WGO and/or their combination with $CdCl_2$ showed amelioration in such external abnormalities compared to $CdCl_2$- treated group.

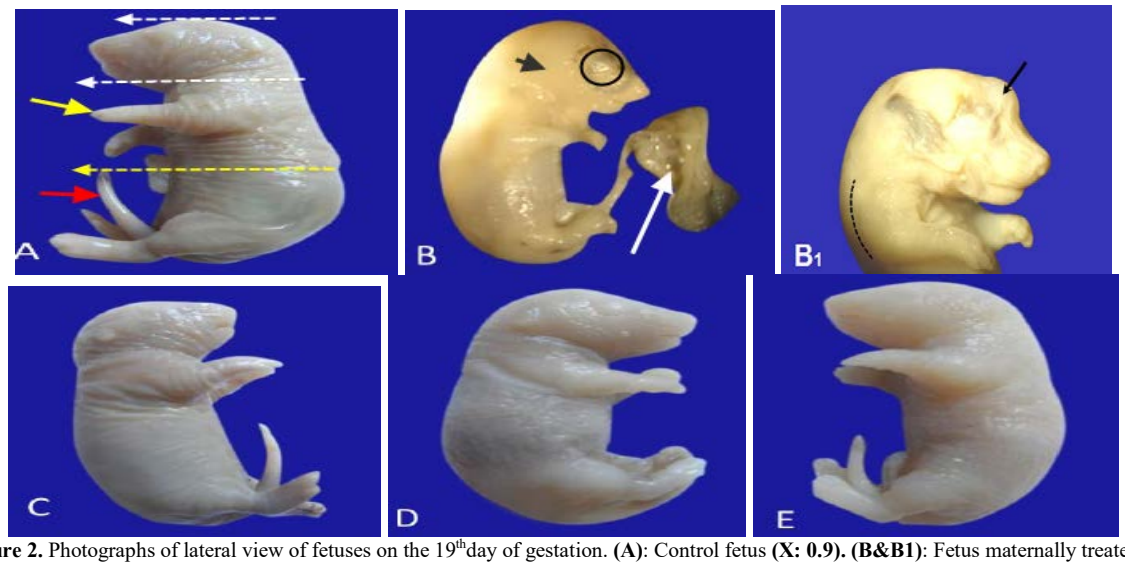
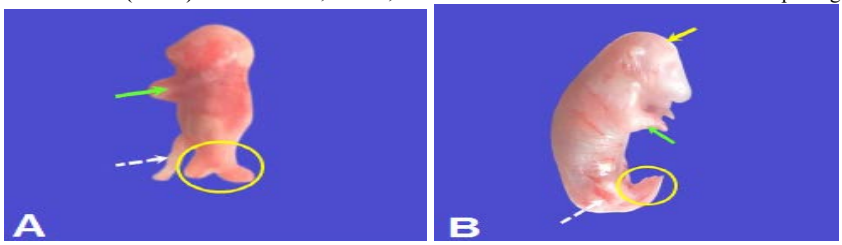
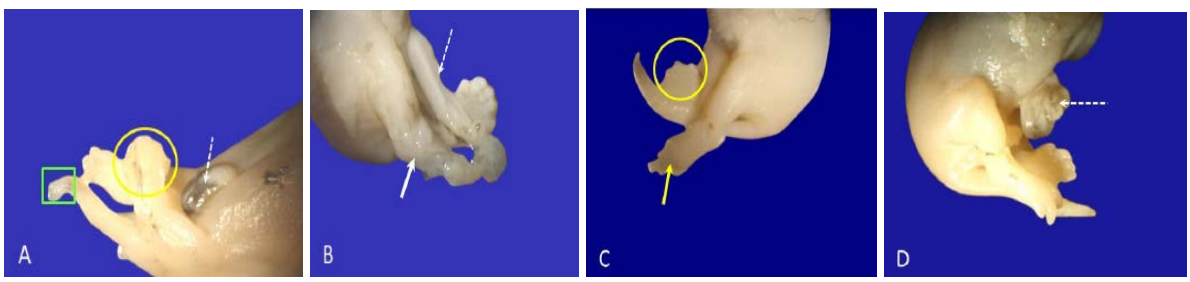



**Figure 2.** Photographs of lateral view of fetuses on the 19thday of gestation. **(A)**: Control fetus **(X: 0.9). (B&B1)**: Fetus maternally treated with CdCl2 showed growth retardation, exophthalmia and open eyelid (black circle) microtia (arrow head), short fore and hind limbs and umbilical hernia (white arrow), exencephaly (black arrow) and abnormal bending of the body (dotted black line) **(X: 1.4&1.6)** respectively**. (C):** Fetus maternally treated with CdCl2 + Vit. E **(X: 0.9). (D):** Fetus maternally treated with CdCl2+ WGO **(X: 0.9). (E):** Fetus maternally treated with CdCl2+ WGO +Vit. E **(X: 0.9).** Notice: In C, D & E; the fetuses more or less have a normal morphological appearance.




**Figures (3):** Photographs of fetuses maternally treated with cadmium chloride on the 19thday of gestation showing gross morphology. **(A)** Fetus shows atrophy in the forelimb (green arrow), umbilical hernia (dotted white arrow) and syndactayly (yellow circle) **(X:1.2). (B)** Fetus shows exencephaly (yellow arrow), short fore limbs (green arrow), atrophy in left hind limb (dotted white arrow), right hind limb club foot (yellow circle) and slight bending of the body **(X:1.3)**




**Figures (4):** Photographs of fetuses maternally treated with CdCl2 on the 19th day of gestation, showing different types of malformations in the posterior region **(X:1.5). (A)** Umbilical hernia (dotted white arrow), syndactayly (yellow circle), kinky tail (green square). **(B)** Abnormal hind limb without distinct toes (white arrow). **(C)** Abnormality of the foot region (yellow arrow). **(D)** Umbilical hernia (dotted white arrow)

## 4. Discussion

Cadmium is one of the most dangerous occupations and environmental toxins. It promotes an early oxidative stress and contributes to the development of serious pathological conditions (Jama *et al.*, 2013). Furthermore, cadmium has been shown to be both embryotoxic and teratogenic in different animal species, take place as a consequence of cadmium given in early pregnancy (EL-Sayed *et al.*, 2013).

The present anemic status could be attributed to a reduction in the rate of the erythrocyte formation through hypo-induction of erythropoietin in the kidneys after long-term of cadmium exposure (Onwuka *et al*., 2010). Furthermore, cadmium intoxication occurred by loss of cell membrane integrity, shortened life span of erythrocytes and occurrence of anemia (Attia *et al*., 2013). These findings were consistent with a previous study by Goncalves *et al.* (2009) who reported that treatment with cadmium chloride caused a r eduction in hematological parameters as a result of er ythrocyte destruction. Exposure to Cd $Cl_2$ induced oxidative damage of erythrocytes leading to an observation of normocytic normochromatic anemia and lymphopenia (Hassan *et al.*, 2012). Al-Asgah *et al.* (2015) reported that the decrease in WBCs count might be the consequence of Cd-induced lipid peroxidation and damage of their cell membrane. The liver, spleen and bone marrow are the major hematopoietic organs which are considered as targets of Cd exposure (Amara *et al.*, 2008). Moreover, cadmium may inhibit heme synthesis by decreasing the absorption of iron from the gastrointestinal tract (Elsharkawy and El-Nisr, 2012).

The improvement of hematological parameters in Vit. E and/or WGO treated groups might be due to increase the coronary and peripheral blood circulation also, vitamin E is a h ighly effective fat-soluble vitamin with a variety of cellular membrane stabilizing-antioxidant functions. Vitamin E has been suggested to prevent the oxidation of polyunsaturated fatty acids in Red Blood Cell (RBC) membrane, thus inhibiting the premature erythrocytelysis. Animal studies have shown that treatment with vitamin E enhanced erythropoiesis and improved blood hemoglobin levels in these animals (Jilani and Iqbal, 2011) Moreover, wheat germ oil contains some B complex vitamins ($B_6$, $B_{12}$ and folic acid) which are essential for the formation of red blood cells and acts as anti-inflammatory (Abdel-Fattah *et al.*, 2011).

The increase in serum AST and ALT activities of maternal and their fetuses reflected the disturbances in hepatic function after cadmium administration during the gestation period. These results were in agreement with the result of Heydamejad *et al*. (2013). The main mechanism involved in Cd hepatotoxicity, its binding to sulfhydryl group in mitochondria and the initiation of inflammation. Also, oxidative stress, due to decrease in antioxidative capacity, plays a role in chronic Cd hepatotoxicity. Khalifa *et al*. (2011) stated that the results of the his study indicated that Wheat Germ Oil (WGO) significantly reduced the toxic effects of chlorpyrifos by altering the hepatic enzyme activities and thus they can be considered a potential hepatoprotective agent in conditions of organophosphate poisoning.

The elevation in serum concentrations of urea and creatinine in maternal and their fetuses of Cd –treated group could be considered as a reflection of deteriorating renal performance. Chronic Cd exposure can cause renal proximal tubular dysfunction resulting from the release of Cd Metallothionein (MT) from the liver and its accumulation and degradation in the renal tubular epithelial cells, inducing proximal apoptosis in different cell types (Tarasub *et al*., 2011). Buha *et al*. (2012) reported that cadmium was highly accumulated in kidney of animals exposed to it via oral routes. These results were in the same line with Wang *et al.* (2010) who suggested that the elevation of urea and creatinine concentrations could be attributed to the degenerative changes in the lining epithelial cells of renal tubules.

This indicated that vitamin E and wheat germ oil significantly reduce the toxic effect of cadmium by improving the hepatic enzyme activities in serum and thus can be considered a p otential hepatoprotective agent (Ahmed *et al*., 2013) These results were in agreement with Megahed (2011) who demonstrated that the supplementation of vitamin E and wheat germ oil caused an improvement in the levels of creatinine and urea as well as AST and ALT activities.

The elevation in MDA and decline in GSH levels in liver and kidney of the $CdCl_2$ maternal treatment and their fetuses might be attributed to cadmium induced oxidative stress in tissues by increasing lipid peroxidation and altering the antioxidant status in liver and kidney tissues (Rajasekaran and Periasamy, 2012). The depletion of cellular glutathione could be explained through the exhaustive use of GSH in conjugation to cadmium catalyzed by Glutathione-S-Transferases (GST) (Sarkar *et al*., 2013).

In general, mechanisms by which Cd can induce oxidative stress through free radicals over production and the disruption of the mitochondrial membrane which appear to be the primary target of its cellular effect (Thompson and Bannigan, 2008). These results came accordance with previous studies, which reported that Cd exposure resulted in GSH depletion and increased MDA level in kidney and liver cells in maternal and their fetuses (Al-Attar, 2011).

The present depletion in liver and kidney antioxidant enzyme activities of $CdCl_2$ maternal treated group and their fetuses might be due to cadmium i nduced cell membrane damage and alterations in dynamic permeability of membranes, which was followed by the release of intracellular enzymes to the blood stream or might be attributed to their utilization by the enhanced production of ROS (Ho *et al*., 2013). These results were in agreement with the study of Lakshmi *et al.* (2012). They showed a decrease in the activity of hepatic catalase and glutathione peroxidase in cadmium-maternal treated animals.

Pregnancy itself is a stressful condition in which many physiological and metabolic functions can be altered to a considerable extent against the increase in reactive oxygen species (ROS) during pregnancy and protect the fetus. The presence of heavy metals in the placenta may be detrimental for placental SOD and GPx activities and, as a result, the fetus is subject to some degree of oxidative stress which may result in potential damage. In addition, oxidative stress influences both implantation and early

development which decides a successful pregnancy (Lee *et al.*, 2009).

Vitamin E and wheat germ oil supplements have significantly minimized the severity of lipid peroxidation, and enhanced the activities of antioxidant enzymes as well as reduced GSH level in hepatic and renal tissues of maternal and their fetuses. These results were in agreement with Layachi and Kechrid (2012). This effect could be due to vitamin E, a strong lipid soluble antioxidant present in the cell, naturally accumulates in the membranes of mitochondria, endoplasmic reticulum and protects liver and kidney cells from lipid peroxidation. Furthermore, Abdul-Hammid *et al.* (2004) reported that co-administration of wheat germ oil caused amelioration in the antioxidant enzymes and reduced pe roxidative process. This may be due to vitamin E in wheat germ oil which is a potent peroxyl radical scavenger that prevents the propagation of free radical damage in biological cell membranes.

Cd administration could be teratogenic or fetotoxic depending on the dose, chemical species and administration during gestational period (Salvatori *et al.*, 2004). The reduction in the number of viable fetuses might be explained on the basis of incomplete formation of the placenta and degeneration of the trophoblast and decidual cells, which play an important role in the transmission of nutrients to the embryo (Aboubakr *et al.*, 2014). Also, they added that the fetotoxicity, high resorption ratio and fetal loss and malformations could be attributed to the inhibition of DNA transcription in the rapidly divided fetal cells.

It has been observed that the placenta is a natural defense against Cd toxicity during pregnancy, because it acts as a barrier for Cd transfer from mother to fetus by sequestering its excess from the blood and minimizing its transfer to the fetus (Sorkun *et al.*, 2007). In addition, it has been reported that cadmium is bound to MT in the placenta, while the placenta functions as a partial barrier for cadmium between maternal and fetal blood. Also, García and González (2010) stated that cadmium reaches the placenta or embryo at organogenetically sensitive time ($9^{th}$ day of gestation) in *Wistar* rats.

Resorption and fetuses lethality might be attributed to the inhibitory action of $CdCl_2$ on the protein synthesis, placental dysfunction and/or Cd intoxication to heart, kidney and liver (Ji *et al.*, 2011). Cd is an endocrine disrupter with detrimental effects on mammalian reproduction the hypothesized that Cd disrupts the proliferative growth and physiological function of placental trophoblasts cells in rat, depending on the studies which showed that cadmium has potent estrogen-and androgen receptors (Sekhon *et al.*, 2010). The reduction in the body weight and length of fetus maternally treated with $CdCl_2$ might be due to an impairment of blood flow to the placenta and reduced uterine transfer of nutrients and oxygen to the fetal circulation. So, these results coincide with the previous studies of Shirai *et al.* (2010). Llanos and Ronco (2009) reported that the fetal growth restriction could be related to impaired placental function due to toxic metals, thus inhibiting the appropriate transfer of essential nutrients to the fetus, which are indispensable for life maintenance and normal development and growing.

Furthermore, this reduction could be explained by the fact that cadmium may devastate the placental function through its congestive effect and hyaline degeneration as well as thrombus formation of its vessels, thus interfering with the transport of amino acids necessary for normal growth of the fetus. Also, the direct cytotoxic effect of cadmium on the fetal tissue may lead to decline in fetuses's size (Ji *et al.*, 2011).

The obtained results revealed that cadmium toxicity produced multiple external deformities in the fetus like exencephaly. This may be due to neural tube defects during neurulation and/or due to $CdCl_2$ exposure before neurulation caused an opening in the anterior neural pore, anophthalamia, microphthalmia may be due to reduced thickness of the neuroblastic layer, neurosis or pyknosis of retinal cells (Yang *et al*., 2006).

Fetuses maternally treated with $CdCl_2$ showed deformities in limbs such as clubfoot. Club foot formation might be due to: 1- indirect action of metal, 2- alteration of maternal physiology, which disturbs the hormonal balance in mother or 3- direct effect on the tissue primordial of foot. Moreover, syndactayly and amelia might be via reduction in cell proliferation of the distal margin and inhibition of chronic genesis (Behbahani *et al*., 2014).

Also, the occurrence of open eyelids in the present study after $CdCl_2$ mother administration might be due to partial ossification of dermal bones that may have affected the diameter of eye orbit. This alteration could result in changes in the attachment of eye muscles, thereby leading to the condition of open eyes.

In addition, umbilical hernia, abnormal bending of the body, short and absent tail; microtia in fetuses maternally treated with $CdCl_2$ were observed. These results were in agreement with the results of El-Sayed *et al.* (2013) who reported that Cd toxicity produced multiple external deformities in the fetus like exencephaly, micrognathia, ablephary, microphthalmia, short and kinky tail and clubfoot.These malformations could be due to the genotoxic effects of cadmium that produced breakdown of the DNA and DNA protein cross link, thus interfering with normal formation of different parts of the body and/or transport of even smaller quantities of the metal into the embryo during early gestation could cause severe malformations (Velázquez *et al*., 2013). The increase in ROS also involved in defective embryo development and the retardation of embryo growth, which is attributed to cell membrane damage, DNA damage and apoptosis (Ronco *et al.*, 2011). The results of Dı´az *et al*. (2014) revealed a clear embryotoxic and a teratogenic effect of Cd, the former as a significant increase in the number of resorptions, and the latter as a significant decrease of the gestational sac weight, and the size and weight of foetuses of Cd-treated dams as well as induced malformations in skull bones, vertebrae and thoracic, and pelvian limbs.

In the present study, supplementation of vitamin E, wheat germ oil or their combination with cadmium chloride more or less prevented fetal malformations and fetal resorption also, improved body growth and bone formation. These results were in agreement with Delashoub and Khojasteh (2012) who reported that co-administration of Vit. E reduced oxidative stress induced Intrauterine Growth Retardation (IUGR) and reversed metal induced growth retardation. Vitamin E has a protective effect against source of free radical in pregnant rats. Also, vitamin E has increased the status of fertility

and percentage of normal pups born from the metal exposed rats. Vitamin E supplementation may also play a role in fetal growth, as shown previously that body length was found to be positively associated with maternal-tocopherol concentration which indicated that maternal vitamin E h elped in fetal growth (Ammar *et al*., 2009). Also, those stated that vitamin E may be beneficial in preventing fetal malformation and fetal resorption due to its antioxidant potency (Ahmed *et al.*, 2013).

The present results are in the same line with the results of Abd El- Aziem *et al.* (2005) who suggested that wheat germ oil contains high levels of essential fatty acids, which the body does not naturally produce. So, for this reason, these fatty acids need to be ingested in order to stimulate cell regeneration and growth also, to maintain a h ealthy immune system, reproductive system, nervous system, and cardiovascular system. It has been proven that using wheat germ oil is more effective in delivering results than using synthetic varieties of vitamin E. Moreover, the presence of different types of fatty acids in wheat germ oil caused increasing the stability of the genetic protein P53, which is a critical factor in the reduction of cell mutation as regulates tumor necrosis factor, regulation of both normal embryonic development and prevention of developmental defects after teratogenic exposure (Omima *et al.,* 2011).

## 5. Conclusion

The present results revealed that the supplementation with vitamin E, wheat germ oil and their combination during $CdCl_2$ exposure, showed an antioxidant activity and a protective effect against $CdCl_2$ induced hematological, hepatic and renal toxicities, fetal growth retardations and malformations. However, future studies may be able to ensure many mechanisms involved in the beneficial effect of vitamin E and wheat germ oil against $CdCl_2$ induced deleterious effects.

## 6. Conflict of interest

The authors declare that they have no conflict of interest.

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JJBS
Jordan Journal of Biological Sciences

Volume 10, Number 1,March 2017
ISSN 1995-6673
Pages 49 - 55

# Toxicity of N-alkyl Derivatives of Chitosan Obtained from Adult of *Chrotogonus trachypterus* (Orthoptera, Acrididae) against the Wheat, Cabbage and Oleander Aphid (Hemiptera: Aphididae) Species

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*Received: December 17, 2016 Revised: January 30, 2017 Accepted: February 5, 2017*

**Abstract**

Chitosan and its derivatives have received attention as alternatives to pesticides in agriculture. Insects are a good source for chitosan isolation. In the present study, chitosan was obtained from *Chrotogonus trachypterus* (Orthoptera, Acrididae), and its N-alkyl derivatives were synthesized. Experiments were conducted to assay their aphicidal activity against three aphid species. All derivatives had a higher aphicidal action (> 98%) than pure chitosan (> 15.2%) against aphid species. N-(3-phenyl butyl) chitosan and N-(ethyl butyl) chitosan were the most and least active derivatives. Results confirmed that the chemical modification of chitosan increased the aphicidal activity. A comparison of the aphicidal activities confirmed that N-alkyl derivatives of commercial chitosan had more toxic effects on aphid species than derivatives of grasshopper chitosan. No significant differences were observed between the two groups of commercial and *C. trachypterus* chitosan derivatives. This encourages us to introduce N-alkyl derivatives of grasshopper chitosan as a promising alternative source of aphicides in future.

**Keywords:** Chitosan, N-alkyl derivatives, Insecticide, *Aphis nerii, Schizaphis graminum, Brevicoryne brassicae, Chrotogonus trachypterus*.

## 1. Introduction

Chitin is a polymeric component present in the skeletal structure of arthropods, algae, crustaceans and fungi (Podile and Neeraja, 2011). Chitin and cellulose are two linear biopolymers; in their chemical structures, monomeric units of *N*-acetyl-2-amino-2-deoxy-d-glucose are connected by β-(1-4)-glycosidic bonds. Chitin has been extracted and characterized from a limited number of insects such as Lepidoptera (Zhang *et al*., 2000; Paulino *et al*., 2006), Hymenoptera (Nemtsev *et al*., 2004; Majtan *et al*., 2007; Marei *et al*., 2015; Kaya *et al*., 2016), Diptera (Ai *et al*., 2008), Homoptera (Sajomsang and Gonil, 2010), Coloeptera (Marei *et al*., 2015; Liu *et al*., 2012), and Orthoptera species (Marei *et al*., 2015; Kaya *et al*., 2014b; Kaya *et al*., 2015c).

Due to the insolubility of chitin in most solvents, modifications of chitin's structure are conducted to obtain its derivatives, such as chitosan, which, in ambient conditions, is more soluble in water and dilute aqueous organic acids, like acetic acid and formic acid (Toffey *et al*., 1996). Chitosan is formed by partial deacetylation of chitin, and preparation of its derivatives results in improved solubility in general solvents (Liu *et al*., 2012). Currently, commercial chitosan is primarily obtained from crustaceans, such as crab, and shrimp. However, such sources are unavailable in arid and semi-arid areas. Thus, finding new sources of chitosan is important, and pest insects may be a promising source to this end.

Previous researches suggested that reductive alkylation of chitosan with aldehydes or ketones could result in the interesting biological activities against some insect pests (Rabea *et al*., 2003; Rabea *et al*., 2006). In the present study, we obtained natural chitosan by deacetylation of chitin that was extracted from the grasshopper *Chrotogonus trachypterus* (Orthoptera, Acrididae). This grasshopper is found abundantly in the Sistan region (Zabol, Iran) and causes economic losses of seedlings of barley, wheat and vegetables. The present study aims to

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synthesize N-alkyl derivatives of chitosan of both commercial (low molecular mass) and *C. trachypterus* sources and to examine their insecticidal activities on aphids including *Aphis nerii* Boyer de Fonscolombe, *Schizaphis graminum* Rondani, and *Brevicoryne brassicae* Linnaeus.

## 2. Materials And Methods

### *2.1. Chemicals*

Low molecular weight ($3.60\times10^5$ g/mol) chitosan and all chemicals were purchased from Sigma Aldrich (Spain) and used without further purifications.

### *2.2. Isolation of Chitin from Adult of C. trachypterus*

Adults of *C. trachypterus* were captured in April-July 2015 in wheat fields of Sistan region (Sistan va Baluchestan, Zabol, Iran). The adults of grasshoppers were starved for 48 hours to eliminate their gut contents and were then killed by freezing at -20 °C. The killed specimens were washed with distilled water and dried at room temperature. The samples were air-dried at 50°C for two days. Then, the air-dried specimens were pulverized using a mortar and stored at 4°C. In the step of demineralization, 5 grams of the powdered grasshoppers were treated with 1 M HCL (250 mL, 60 min, 75°C). The demineralized samples were washed and filtered several times to reach neutrality. The next step was deproteinization, in which the samples were treated with 1 M NaOH (250 mL, 24 h, 80°C), followed by filtering and washing with distilled water to obtain a neutral pH. The samples were decolorized by treating the precipitate with 1% potassium permanganate (100 mL, 2 h). The obtained light chitin was rinsed with distilled water several times to reach neutrality and was dried in an oven (24 h, 50°C) (Kaya *et al*., 2014a).

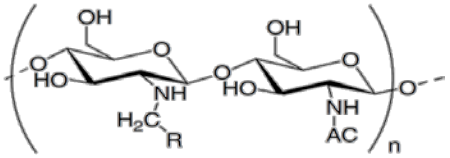
### *2.3. Chitosan Preparation*

The purified chitin was refluxed in 50% NaOH (100°C, 5 h). The samples were rinsed many times until neutralization. The chitosan samples were dried at 50°C (24 h). To purify, the chitosan structures were dissolved in 2% acetic acid and re-precipitated in 20% NaOH. The chitosan samples were washed to obtain a neutral pH. The described potentiometric titration by Abdou *et al.* (2008) and Marei *et al*. (2015) was used to measure the degree of deacetylation (DD) of *C. trachypterus* chitosan (Ct.c). Furthermore, the molar mass of Ct.c was determined using a solvent system (0.2 M of NaCl, 0.1 M of acetic acid), as described by Erdogan and Kaya (2016). The molar mass of Ct.c was determined with data on the intrinsic viscosity and using the Mark-Houwink equation (Wang *et al*., 1991).

### *2.4. Synthesis of N-alkyl Chitosan Derivatives*

The N-(alkyl) derivatives of both commercial and *C. trachypterus* chitosans (Cc and Ct.c, respectively) were synthesized using the method described by Kim *et al*. (1997). Eighteen nmol of chitosan (3 grams calculated as glucose amine unit) was dissolved in 300 mL 1% (v/v) glacial acetic acid. One equivalent of aromatic aldehydes (2-ethyl butyraldehyde, n-tridecanal, phenyl acetaldehyde, diphenyl acetaldehyde, 3-phenyl butyraldehyde) was separately added to the chitosan solution while stirring for 60 minutes at room temperature. Next, 1 M aqueous NaOH was added drop-wise to adjust the solution's pH to 4.5. 10% (w/v) NaBH4 (1.5 equivalents to the aldehyde) was added to this solution and stirred for 90 minutes at room temperature. To precipitate N-(alkyl) derivatives, the pH of the solution was adjusted to 10. The precipitate was neutralized by rinsing it with distilled water many times. Finally, the precipitates of N-(alkyl) derivatives were soxhlet-extracted with 1:1 (v/v) ethanol/diethyl ether for 48 hours, and the residues were oven-dried overnight at 60°C. Table (1) shows a series of *N*-Alkyl Chitosan (NAC) derivatives with their chemical structure.

**Table 1.** Chemical structure of NAC derivatives




| Compound abbreviation | R | Compound name |
|---|---|---|
| NAC-1 | $CH_3CH(C_2H_5)CH_2$ | N-(ethyl butyl) chitosan |
| NAC-2 | $CH_3(CH_2)_{11}$ | N-tridecanyl chitosan |
| NAC-3 | $(C_6H_5)CH_2$ | N-(2-phenyl ethyl) chitosan |
| NAC-4 | $(C_6H_5)_2CH$ | N-(2, 2-diphenyl ethyl) chitosan |
| NAC-5 | $CH_3CH(C_6H_5)CH_2$ | N-(3-phenyl butyl) chitosan |

### *2.5. Aphid Sampling and Rearing*

To initiate the aphid cultures, the aphids (*A. nerii*, *S. graminum*, and *B. brassicae*) were originally collected from randomly selected fields in the suburbs of Zabol, Iran during spring 2015. The aphid colonies were reared in a $20\times15\times10$ $cm^3$ container under constant temperature in greenhouse conditions (26±2°C, 65±5% RH, 16:8 L:D). Aphids were reared for 2-3 generations (Wille and Hartman, 2008) in the laboratory before the insecticidal tests were carried out.

### *2.6. Bioassay Tests*

Leaf-dip and plant systemic methods developed by Badawy and El-Aswad (2012) were used to assay the insecticidal activities of chitosan derivatives. N-alkyl chitosan derivatives of Cc and Ct.c were dissolved in 0.5% (w/v) aqueous acetic acid (50 mL). Then, a series of concentrations (200, 400, 600, 800, and 1000 mg/L) were prepared by dilution of the stock solutions.

In the leaf-dip method, fresh leaves of host plants of each aphid species were dipped in the chitosan derivatives for 30 seconds. The treated leaves were air-dried at room temperature (30-60 minutes) and then placed petri dishes (9 cm diameter) on filter papers (Whatman no. 1). A fine brush was used for transferring 25 wingless adult aphids from a stock culture to each petri dish. In the control, the leaves were treated with distilled water. The treatments were kept at 26±2°C, 65±5% RH and 12:12 L: D. Aphid mortality was recorded at 24 and 48 hours post treatment. Aphids, which were unable to move after the treatments, were scored as dead (Badawy and El-Aswad, 2012).

To study the insecticidal effects of chitosan derivatives on *A. nerii*, in the plant systematic method, branches of oleander plant were put in conical flasks containing the experimental concentrations (200, 400, 600, 800, and 1000 mg/L) of the derivatives. In addition, to assay the insecticidal activity of chitosan derivatives on *S. graminum*, and *B. brassicae*, the different concentrations of chitosan derivatives were added to hydroponically grown wheat (Moon *et al*., 1995). Thirty newly matured females of aphid species were transferred from the stock culture on the upper side of the leaves of the plant hosts (Badawy and El-Aswad, 2012). Distilled water was used in the control treatment. The treatments were kept under the conditions described above. All experimental bioassays were repeated for three replications.

To compare the efficiency of Cc and Ct.c, all experiments were first conducted with a s eries of Ct.c concentrations. Afterward, the maximum and minimum percentages of aphid mortalities with concentrations of Ct.c were obtained. The same concentrations of NAC derivatives were synthesized and tested on the aphids.

### *2.7. Statistical Analysis*

Under a co mplete randomized design, data were compared by one-way analysis of variance (ANOVA) using SPSS (Statistical Package for Social Sciences, USA) software (version 21.0). Differences between treatment means were established using Student- Newman-Keuls (SNK) test (Snedecor and Cochran, 1989). Differences at $p \leq 0.05$ were considered to be statistically significant.

## 3. Results

### *3.1. Chitin and Chitosan Characterization*

An amount of 9.1% Chitin was isolated from adult of *C. trachypterus.* Degree of deacetylation (DD) of chitosan from *C. trachypterus* was found to be 97%, which is considerably pure for chitosan. In addition, chitosan samples obtained from adult *C. trachypterus* had a m olar mass of 8.1 kDa.

### *3.2. Insecticidal Activities of N-alkyl Chitosan Derivatives*

Table (2) shows the insecticidal activity of N-alkyl derivatives of *C. trachypterus* chitosan against the tested aphid species using the leaf dip and systemic methods. Control CA (chitosan and acetic acid) showed 2.3-10.2% and 6.4-15.2% mortality in leaf dip and systemic bioassays, respectively. Mortalities of 0.0-4.1% were observed when aphides were treated with CW (chitosan and distilled water). Likewise, chitosan derivatives that had their NH2 group substituted with an alkyl group exhibited a range of aphicidal activity between 10.3-98.9% against the treated aphid species (Table 2). Our results showed that *N*-(3-phenyl butyl) chitosan (NAC-5) was the most active derivative in the leaf dip (67.1%, and 89.2% after 24 and 48 h of treatment, respectively) method, as well as in the systemic method (98.9%) against *B. brassicae* at 1000 mg/L. In addition, *N*- tridecanyl chitosan (NAC-2) showed a mean mortality of 47.5%, 68.3%, and 88.87% against all treated aphid species in the leaf dip (24 and 48 h post treatment) and systemic bioassay methods, respectively. *N*-(2, 2- diphenyl ethyl) chitosan (NAC-4) and *N*-(2-phenyl ethyl) chitosan (NAC-3) showed moderate aphicidal activity with a r ange 34-67% in both bioassay methods. While *N*-(ethyl butyl) chitosan (NAC-1) was found to be the least efficient for killing the aphids in the leaf dip method (26.5-47.5% 24 a nd 48 h post treatment, respectively), its systematic effect on aphids interestingly showed mortalities higher than 69% at 1000 mg/L.

**Table 2.** Aphicidal activity of N-alkyl derivatives of chitosan obtained from adults of *Chrotogonus trachypterus* against aphid species

| Treatment | Concentration | Leaf dip method 24 h | | | Leaf dip method 48 h | | | Systemic effect method 48 h | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | mg/L | Mortality % ± SE | | | Mortality % ± SE | | | Mortality % ± SE | | |
| | | *Aphid 1* | *Aphid 2* | *Aphid 3* | *Aphid 1* | *Aphid 2* | *Aphid 3* | *Aphid 1* | *Aphid 2* | *Aphid 3* |
| CW | 0 | 0.0±0.0 l | 0.0±0.0 l | 0.4±0.0 m | 1.00±0.4 m | 2.5±0.01 n | 1.8±0.4 l | 2.9±1.21 i | 4.1±0.5 j | 3.0±0.9 i |
| CA | 0 | 2.3±1.2 k | 5.6±0.0 k | 4.5±1.1 kl | 3.7±0.3 l | 10.2±2.5 m | 9.8±0.1 jk | 6.4±2.2 h | 14.4±1.8 i | 15.2±0.7 h |
| NAC-1 | 200 | 10.3±3.2 ij | 12.7±2.2 ij | 8.1±0.5 k | 18.6±1.8 jk | 20.3±0.8 jk | 13.0±2.0 ij | 51.0±0.0 e | 57.4±1.1 ef | 55.1±0.1 e |
| | 400 | 10.6±5.0 ij | 19.4±4.1 hi | 10.5±0.0 jk | 21.1±0.0 jk | 26.4±1.4 j | 15.1±0.3 ij | 50.2±1.3 e | 61.2±0.0de | 56.3±1.4 e |
| | 600 | 15.1±2.4 hi | 21.0±1.8 hi | 12.9±1.4 jk | 33.2±1.5 ij | 41.1±0.0 fg | 18.0±0.5 ij | 54.0±1.0 e | 62.3±2.4de | 57.5±0.5 e |
| | 800 | 24.0±1.9efg | 26.2±1.9gh | 20.1±0.0 hi | 38.0±2.1ghi | 49.6±2.4def | 24.3±1.1 i | 60.3±0.0 d | 68.0±0.0 c | 63.7±0.0cd |
| | 1000 | 25.5±2.8efg | 30.2±3.0 f | 24.0±0.6gh | 46.0±1.0 fg | 56.8±1.9 d | 39.9±0.9 fg | 72.9±2.1bc | 75.9±0.1 c | 69.0±0.3bc |
| NAC -2 | 200 | 19.3±1.2 gh | 21.4±0.9 hi | 17.0±1.7 ij | 31.4±0.0 ij | 49.0±0.2def | 32.2±2.1gh | 60.0±1.2 d | 70.9±1.1 c | 51.7±0.0 f |
| | 400 | 23.0±0.8efg | 31.9±2.0 f | 25.4±0.0gh | 40.9±2.1gh | 52.7±0.0 de | 39.7±0.5 fg | 73.9±1.5bc | 74.0±1.5 c | 59.0±0.6de |
| | 600 | 30.5±0.0 e | 39.0±2.7de | 39.2±2.2cd | 50.0±1.5 ef | 61.7±1.1 c | 43.7±0.0 f | 75.0±0.0bc | 76.5±2.0 c | 63.1±0.5cd |
| | 800 | 33.7±0.1 de | 46.0±1.4cd | 49.0±3.0bc | 55.8±1.3 de | 66.1±2.3 c | 50.0±1.4de | 84.7±0.1 b | 83.5±0.0 b | 75.0±1.3bc |
| | 1000 | 40.9±3.5 c | 50.8±2.1bc | 50.8±0.4 b | 63.7±0.0 cd | 72.3±0.0 b | 68.9±0.1bc | 85.6±0.0 b | 94.0±2.3 a | 87.0±0.1ab |
| NAC-3 | 200 | 12.6±1.2 hi | 13.0±1.1 ij | 15.0±0.9 jk | 29.9±0.0 ij | 32.6±1.5 hi | 30.8±0.9gh | 45.9±1.7 ef | 50.0±0.5 fg | 50.1±1.1 f |
| | 400 | 18.3±2.6 gh | 22.5±0.3 hi | 20.9±2.1 hi | 37.5±1.4 hij | 39.5±1.5 h | 35.5±1.2 fg | 50.8±2.1 e | 52.5±1.7 fg | 54.6±0.7 e |
| | 600 | 26.2±2.1efg | 25.1±0.1gh | 21.8±3.0 hi | 41.1±0.5 gh | 44.0±2.1 ef | 36.0±1.0 fg | 51.0±0.0 e | 59.4±2.0 ef | 62.8±0.3cd |
| | 800 | 31.7±1.4 de | 29.6±0.0 fg | 32.1±0.0 ef | 50.3±0.5 ef | 63.0±0.0 cd | 43.1±0.5 f | 52.8±0.5 e | 64.4±0.5de | 70.9±0.1bc |
| | 1000 | 31.9±0.9 de | 32.4±0.1 f | 38.4±1.9cd | 59.4±2.2 d | 69.6±0.9 c | 51.3±0.0de | 61.9±3.0 d | 67.5±0.5 d | 74.2±0.0bc |
| NAC-4 | 200 | 13.2±0.1 hi | 15.1±0.5hij | 15.5±2.0 jk | 30.6±2.1 ij | 42.8±0.7 fg | 34.4±0.0 fg | 35.9±1.7fg | 49.0±0.5gh | 44.1±1.1 g |
| | 400 | 15.0±2.0 hi | 18.0±1.4 hi | 17.1±1.1 ij | 39.5±0.3 gh | 44.0±1.4 ef | 40.1±0.0 fg | 40.8±2.1 f | 52.5±1.7 fg | 50.6±0.7 f |
| | 600 | 15.6±3.1 hi | 28.5±0.0gh | 25.5±1.9gh | 42.8±0.0 gh | 52.1±2.1 de | 50.5±0.5de | 41.0±0.0 f | 59.4±2.0 ef | 54.8±0.3 e |
| | 800 | 29.1±0.9 e | 35.6±0.5 ef | 30.4±0.5 ef | 52.1±1.4 ef | 67.0±0.0 c | 55.2±1.7 d | 42.8±0.5 ef | 64.4±0.5 d | 60.9±0.1cd |
| | 1000 | 37.5±4.1 d | 40.5±0.1de | 41.7±0.0cd | 60.0±0.9 d | 72.2±3.8 b | 62.5±1.2 c | 54.9±3.0 e | 67.5±0.5 d | 64.2±0.0cd |
| NAC-5 | 200 | 54.9±4.0 b | 41.5±3.0de | 34.1±1.0de | 67.9±1.9 bc | 62.7±2.4 cd | 55.4±1.2 d | 71.0±2.2bc | 65.2±0.3 d | 61.3±0.2cd |
| | 400 | 57.8±3.0 b | 57.6±1.1 b | 48.2±0.8bc | 70.1±0.0 b | 72.5±0.0 b | 60.0±0.6 c | 79.0±0.0 b | 74.8±0.5 c | 70.1±0.6bc |
| | 600 | 62.0±2.5 a | 59.0±0.0 a | 55.8±0.0 b | 81.0±2.3 a | 77.9±1.5 b | 62.2±0.5 c | 85.1±1.4 b | 84.1±1.0 b | 79.4±0.0 b |
| | 800 | 65.3±1.4 a | 60.2±2.1 a | 59.5±1.4 a | 87.2±1.3 a | 80.0±0.0 a | 72.5±0.0 b | 90.3±0.1 a | 85.6±1.1 b | 80.3±1.1 b |
| | 1000 | 67.1±3.3 a | 62.0±2.3 a | 60.7±0.2 a | 89.2±0.0 a | 81.3±0.2 a | 85.7±1.0 a | 98.9±0.9 a | 92.1±0.9 a | 90.0±1.7 a |
| df | | 26.49 | 26.49 | 26.49 | 26.49 | 26.49 | 26.49 | 26.49 | 26.49 | 26.49 |
| *p*-value | | < 0.0001 | < 0.0001 | < 0.0001 | < 0.0001 | < 0.0001 | < 0.0001 | < 0.0001 | < 0.0001 | < 0.0001 |

*Aphid* ***1***: *Brevicoryne brassicae*; *Aphid* ***2***: *Schizaphis graminum*; *Aphid* ***3***: *Aphis nerii*; CW: chitosan and distilled water; **CA:** chitosan and acetic acid; **NAC-1:** *N*-(ethyl butyl) chitosan; **NAC -2:** *N*-tridecanyl chitosan**; NAC -3:** *N*-(2-phenyl ethyl) chitosan**; NAC -4:** *N*-(2,2-diphenyl ethyl) chitosan **; NAC -5:** *N*-(3-phenyl butyl) chitosan; Data are expressed as mean percentages ±SE of three replicates; Values followed by the same letter within a column are not significantly different ($P \leq 0.05$) according to Student-Newman-Keuls (SNK) test; df – degree of freedom.

## 4. Discussion

### *4.1. Chitin Content (%) of C. trachypterus*

Our results showed that the dry weight of chitin isolated from *C. trachypterus* was 9.1%. In previous studies, chitin content isolated from different insect species varied between 6% and 36%; the maximum chitin yield was obtained from cicada sloughs (Sajomsang and Gonil, 2010). In addition, the dry weights of chitin isolated from *Apis melifera* (Hym. Apidae), *Calosoma rugosa* (Col., Carabidae), and *Holotrichia parallela* (Col., Scarabaeidae) were 2.5%, 5.0%, and 15%, respectively (Marei *et al*., 2015; Liu *et al*., 2012). Kaya *et al*. (2014b) reported that the yield of chitin from seven grasshopper species varied between 5.3% and 8.9%. The desert locust (*Schistocerca gregaria* F., Acrididae) and the Mexican katydid (*Pterophylla beltrani* B. & B., Tettigoniidae) had 12.2% and 11.8% chitin, respectively (Marei *et al*., 2015; Torres-Castillo *et al*., 2015). Kaya *et al*. (2015a) compared chitin structures derived from three *Vespa* species (Hym., Vespidae) and found that the chitin contents of *V. crabro* L., *V. orientalis* L. and *V. germanica* F. were 8.3%, 6.4%, and 11.9%, respectively.

The dry weight of chitin isolated from female and male of grasshopper species, such as *Celes variabilis*, *Decticus verrucivorus*, *Melanogryllus desertus*, and *Paracyptera labiata*, was found to be 4.71–11.84% (Kaya *et al*., 2015c). In addition, it was confirmed that the yield of chitin varied between insect developmental stages and sexes. In the grasshopper *Dociostaurus maroccanus* (Acrididae), the chitin contents of the adults and nymphs were reported to be 14% and 12%, respectively (Erdogan and Kaya, 2016). The adult Colorado potato beetles (*Leptinotarsa decemlineata*, Chrysomelidae) and its larvae reported to yield 20% and 7% chitin, respectively (Kaya *et al*., 2014a). In another study, Kaya *et al*. (2015b) studied the physicochemical properties of isolated chitin from the body of a butterfly species (*Argynnis pandora*, Nymphalidae). The results confirmed that the chitin isolated from the wings was much higher than other body parts, except the wings (22% and 8%, respectively). They hypothesized that the surface morphology of chitin is highly related to the body part of insect.

*4.2. Chitosan Characterization*

Pure chitosan samples (DD= 97%) were obtained from *C. trachypterus*. This reveals that chitin was deacetylated to chitosan. The different DD influences biological, physicochemical and mechanical properties of chitosan. The DD value of chitosan isolated from the nymph of *D. maroccanus* was as 64% and 22%, respectively (Erdogan and Kaya, 2016).

Chitosan samples of *C. trachypterus* had molar mass of 8.1 kDa. Depending on the initial source of chitosan (crab, fungi, insect, shrimp, etc.) and the preparation method, the molar mass (or molecular weight, MW) of chitosan can show a decrease or increase in line with the significant increase or decrease in the degree of deacetylation (reviewed in Yuan *et al*., 2011). Artemia, crab, and shrimp produced chitosan samples with molar mass of 450-570, 483-526, and 2.20 kDa, respectively (Erdogan and Kaya, 2016; Tajik *et al*., 2008; Yen *et al*., 2009; Kucukgulmez *et al*., 2011). In other studies, the molar mass of obtained chitosan from adults of insect pests including *D. maroccanus*, *A. mellifera*, and *L. decemlineata* were found to be 7.2, 200-250, and 2.722 kDa, respectively (Nemtsev *et al*., 2004; Kaya *et al*., 2014a; Erdogan and Kaya, 2016). Chitosan samples with low molecular mass were commonly used in agriculture (gene transferring, plant protection), medicine (biomedical engineering, drug and vaccine delivery), and food production (seed-coating technology) (Erdogan and Kaya, 2016; Yen *et al*., 2009). Thus, it can be suggested that *C. trachypterus* chitosan, like other low molar mass samples, could be used effectively in these areas.

*4.3. Insecticidal Activities of N-alkyl Chitosan Derivatives*

The present study indicate that chitosan, without any substitution, was the least effective among the tested compounds. When chitosan derivatives were assayed, the aphicidal activity increased significantly compared to the control treatments (CW and CA). Chitosan (CA and CW) showed a low insecticidal activity against aphid species, but its chemical modification led to an increase in activity, especially for *N*-(3-phenylbutyl) chitosan (NAC-5) and *N*-tridecanylchitosan (NAC-2). In line with our findings, Rabea *et al*. (2006) demonstrated that *N*-(3-phenylbutyl) chitosan and *N*-tridecanyl chitosan were the most active chitosan derivatives when added to the artificial diet of *Spodoptera littoralis* Boisd (Lep., Noctuidae) larvae. It is suggested that chitosan and its derivatives probably block air from the insect cuticle by forming a layer on that surface, or inducing chitinases' activity in the insect body, thereby causing insecticidal activity.

Despite our findings of low the efficiency of chitosan (CW and CA) against aphid species, Zhang *et al*. (2003) reported that chitosan was an active insecticide against *Plutella xylostella* (Lep., Plutellidae) and homopterous insects with mortalities higher than 70%. Their study demonstrated that the insecticidal activity of chitosan to *P. xylostella* was higher than that of *S. exigua* at 3g/L concentration of chitosan (72% and 40%, respectively). In addition, the mortality of aphid species (*Rhopalosiphum padi* L., *Metopolophium dirhodum* Walker, and *Aphis gossypii* Glover) was 60-80%. Interestingly, in that study, the aphicidal activity was found to be higher than 90% against *Hyalopterus pruni* (Goffroy) on flowers, while *Sitobion avenae* (Fabricius) and *Myzus persicae* (Sulzer) showed a lower susceptibility to the aphicidal activity of chitosan (Yen *et al*., 2009). Similar to their findings, our results showed variable efficiency of NACs against different species of aphids. NAC-5 was insignificantly very potent in killing the treated aphid species (*A. nerii*, *S. graminum*, *B. brassicae*) in the present study. Other NAC derivatives showed a slightly higher effect against *A. nerii*, and *S. graminum* in comparison with *B. brassicae*. The reason that the aphicidal activities of these NACs on *B. brassicae* were lower than those of the same NACs did against *A. nerii*, *S. graminum* is not clear, but it may be because of the powdery cover on the external structure of its body, which might decrease the efficiency of NACs.

In the present study, aphicidal activity was significantly increased in the leaf dip bioassay at 48 h post treatment in comparison to 24 h after treatment. It was of great interest that higher than 88% mortality against aphids was obtained in systemic bioassays with NAC derivatives (NAC-5, NAC-2). The finding of both studies confirmed that in the leaf dip method, aphids feeding on treated leaves for 24 and 48 h were significantly affected; this suggests that oral uptake is essential for aphid control. The aphicidal activity by systemic bioassay confirmed that chitosan derivatives are primarily translocated in the plant phloem, which passively transports mainly water in an acropetal, i.e., upward movement. After the chitosan molecule moved into the plant, the aphids died and the treatments protected the plant (Erdogan and Kaya, 2016).

Rabea *et al*. (2014) showed that chitosan derivatives including N-(4-propyl benzyl) chitosan, *N*-(3,4-methylenedioxy benzyl) chitosan and *N*-(2-chloro, 6-flouro benzyl) chitosan possessed the toxic action of the males and females of *Ceratitis capitata* (Wiedemann, Diptera: Tephritidae) after 24 and 48 h of feeding under laboratory conditions. Time-lapse data of chitosan or its derivatives showed a fair amount of increase in insecticidal activity.

Derivatives of chitosan, including *N*-benzyl, *N*-butyl, *N*-dodecyl, and *N*-octyl chitosan, were evaluated for their activity against *S. littoralis*. Among them, both derivatives of *N*-benzyl chitosan, including *N*-(*p*-isopropyl benzyl) chitosan and *N*-(*o*-nitro benzyl) chitosan, caused significant mortalities of 46%. The most active compound,

*N*-(2-chloro-6-fluorobenzyl) chitosan showed 100% mortality again this pest (Rabea *et al*., 2003).

The minimum and maximum percentages of aphid mortalities when treated with N-alkyl derivative of commercial chitosan (Sigma) demonstrated that due to the initial sources of chitosan, NAC derivatives of Cc (including NAC-1′, NAC-2′, NAC-3′, NAC-4′, and NAC-5′) had more insecticidal effects on aphid species when compared to N-alkyl derivatives of *C. trachypterus* chitosan. Although NAC derivatives of Cc caused a slightly higher percentage of aphid mortalities, the data were statistically insignificant. To the best of our knowledge, there are no studies examining the insecticidal activity of derivatives of chitosan obtained from insect sources. However, if NAC derivatives are to be encouraged and incorporated into pest control, especially for sucking insects, it is important to understand the acute effects that such derivatives may have on the behavior and physiology of insects under greenhouse or field conditions.

## 5. Conclusion

In the present study, chitin and chitosan were derived for the first time from the adults of *C. trachypterus*. The dry weight of the chitin structure of *C. trachypterus* was in the same range as the isolated chitin from other grasshopper species. Because of the large number of individuals in the invasive population, this grasshopper species could be used as a g ood source for chitosan preparation. Degree of deacetylation of *C. trachypterus* was found to be 97%, which was higher than chitosans isolated from other initial sources such as fungi and crustaceans. Our findings suggested that the efficiency of NAC derivatives seems promising because of their more specific mode of action towards aphids, especially by the systemic method of bioassay. It can be suggested that their mechanisms of NAC derivatives on specific pest species require additional study in the future.

## Acknowledgment

The authors would like to thank University of Zabol, Department of Plant Protection and Department of Chemistry for providing facilities.

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JJBS

Volume 10, Number 1,March 2017
ISSN 1995-6673
Pages 57 - 62

Jordan Journal of Biological Sciences

# Growth, Condition, Maturity and Mortality of the Gangetic Leaffish *Nandus nandus* (Hamilton, 1822) in the Ganges River (Northwestern Bangladesh)

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*Received: December 23, 2016 Revised: January 30, 2017 Accepted: February 5, 2017*

**Abstract**

The Gangetic leaffish, *Nandus nandus* (Hamilton, 1822) is an important nutritionally precious food fish in south Asian countries. This is the first inclusive explanation on population parameters of *N. nandus* i.e., population structure (Length-Frequency Distributions, LFDs), growth (Length-Weight and Length-Length Relationships; LWRs, LLRs), condition factors (allometric, $K_A$; Fulton′s, $K_F$; relative, $K_R$; relative weight, $W_R$; and form factor, $a_{3.0}$), reproduction (first sexual maturity, $L_m$) and natural mortality ($M_W$) in the Ganges River, northwestern (NW) Bangladesh. Samples were collected using different fishing gears from April 2014-March 2015. Total Length (TL) and Standard Length (SL) were measured for each individual by digital slide calipers, while individual Body Weight (BW) was weighed by a digital balance. A total of 125 individuals ranging from 3.0-15.1 cm TL and 0.30-52.10 g BW were analyzed in the present study. The 7.00-8.99 cm TL was numerically leading group of the total population. Allometric coefficient (*b*) of LWRs indicate positive allometric growth ($b$>3.0) for TL *vs.* BW and isometric growth ($b$=3.0) for SL *vs.* BW relationship. Additionally, the *b* value for LLR also shows the same growth pattern. In the present study, among four types of condition factors $K_F$ (ranged from 1.258 to 1.336) was best for wellbeing of *N. nandus* in the Ganges River. Wilcoxon signed rank test indicated that, the $W_R$ did not show any significant difference from 100 ($p$ = 0.325), representing the balanced habitat for *N. nandus*. The $a_{3.0}$ was 0.0159 indicating this fish is short and deep in body shape and the $L_m$ was 9.10 (~ 9.00) cm TL and the $M_W$ was estimated as 1.33 $y^{-1}$. The results would be good strategy for conservation of this species in the Ganges River and surrounding ecosystem.

**Keywords**: *Nandus nandus,* Growth, Condition, Maturity, Mortality, Ganges River.

## 1. Introduction

The Gangetic leaffish, *Nandus nandus* is a fresh- and brackish-water benthopelagic species of the family Nandidae. This fish is known as Meni and Bheda in Bangladesh; Nandosh in India and Dalahai in Nepal (Froese and Pauly, 2016). It is distributed throughout the Indian sub-continent including Bangladesh, India, Malaysia, Myanmar, Pakistan, Thailand and Viet Nam (Froese and Pauly, 2016). This fish mainly inhabits streams, rivers, pools, lakes, canals and reservoir (Rainboth, 1996). The *N. nandus* is important for food fish, aquarium trade and they have high market need (Talwar and Jhingran, 1991). However, unfortunately the natural populations are declining fatally due to reckless fishing, habitat destruction (IUCN Bangladesh, 2000; Hossain, 2014; Hossain *et al*., 2015a, b); pollution and other ecological changes to their territory (Mijkherjee *et al*., 2002; Hossain *et al*., 2015c; Hossen *et al*., 2015). As a result, this fish is categorized as vulnerable in Bangladesh (IUCN Bangladesh, 2000) although globally categorized as least concern (IUCN, 2014).

Information on population parameters i.e., growth, reproduction, recruitment as well as mortality of fishes is vital to the implementation of sustainable management strategies for their better conservation (Hossain *et al*., 2009). However, to the best of the authors' knowledge, there are no earlier studies on population parameters of *N. nandus.* Nevertheless, few works on length-weight relationships (Hossain *et al*., 2006), morphometrics and meristics traits (Goswami *et al*., 2007), biology (Das *et al*., 2002), and pathological investigation (Marma *et al*., 2007) have been done.

Therefore, studies on population parameters are immediately needed for proper management policies for this important fishery. Hence, the objective of the present study is to depict the population parameters of *N. nandus*

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including growth pattern (Length-Weight Relationships, LWRs), reproduction (size at sexual maturity, $L_m$), and natural mortality ($M_W$) from the Ganges River, northwestern (NW) Bangladesh using a number of specimens with small to big sizes over a one-year study period. Also, the condition of *N. nandus* was estimated through multi-models.

## 2. Material and Methods

### 2.1. Study Site and Sampling

The present study was carried out in the Ganges River (Lat. 24° 35′ N; Long. 88° 64′ E) NW Bangladesh. A total 125 individuals of *N. nandus* were occasionally collected from the fishermen during April 2014 to March 2015. The fishes were caught using different types of traditional fishing gears i.e., gill net, cast net, square lift net etc. Samples were instantly chilled in ice on site and preserved with 10% buffered formalin upon arrival in the laboratory.

### 2.2. Fish Measurement

Total Length (TL) and Standard Length (SL) were measured to the nearest 0.01 cm using digital slide calipers and total Body Weight (BW) was weighed by an electronic balance with 0.01 g accuracy for each individual.

### 2.3. Growth Pattern

The length-frequency distribution for *N. nandus* was constructed using 1 cm intervals of TL. The growth pattern was determined through LWR with the equation: $BW = a*(TL)^b$ where BW is the total body weight (g) and TL is the total length (cm). The parameters $a$ and $b$ were calculated by linear regression analyses based on natural logarithms: $\ln(W) = \ln(a) + b \ln(L)$. Extremes outliers were deleted from the regression analyses according to Froese (2006). Additionally, on the basis of the $b$ values of LLR (TL *vs.* SL) growth pattern of *N. nandus* was determined.

### 2.4. Condition Factors

The allometric condition factor ($K_A$) was estimated using the equation of Tesch (1968): $W/L^b$, where $W$ is the body weight (g) and $L$ is the TL (cm), and $b$ is the LWR parameter. The Fulton′s condition factor ($K_F$) was calculated using the equation of Fulton (1904): $K_F = 100 \times (W/L^3)$, where $W$ is the body weight (g) and $L$ is the TL in cm. The scaling factor of 100 was used to bring the $K_F$ close to unit. Moreover, the relative condition factor ($K_R$) was calculated following the equation of Le Cren (1951): $K_R = W/(a \times L^b)$, where $W$ is the body weight (g), $L$ is the TL (cm) and $a$ and $b$ are LWRs parameter. For assessing the relative weight ($W_R$), the equation of Froese (2006): $W_R = (W / W_s) \times 100$, were used, where $W$ is the weight of a particular individual and $W_s$ is the predicted standard weight for the same individual as calculated by $W_s = a \times L^b$ where the $a$ and $b$ values are gained from the relationships between TL *vs.* BW.

### 2.5. Form Factor ($a_{3.0}$)

The $a_{3.0}$ of *N. nandus* was calculated according to the equation of Froese (2006) as: $a_{3.0} = 10^{\log a - s(b-3)}$, where $a$ and $b$ are the regression parameters of LWRs and $s$ is the regression slope of *ln a vs. b*. In the present study, a mean slope $S$ = -1.358, was used for estimating the form factor because information on L WRs is not available for this species for estimation the regression (S) of *ln a vs. b*.

### 2.6. Size at First Sexual Maturity (Lm)

The $L_m$ was calculated using the empirical equation, $\log(L_m) = -0.1189 + 0.9157 * \log(L_{max})$, where $L_{max}$ is the maximum observed length (Binohlan and Froese, 2009).

### 2.7. Natural Mortality ($M_W$)

The $M_W$ of *N. nandus* was calculated using the model, $M_W = 1.92 \text{ year}^{-1} *(W)^{-0.25}$ (Peterson and Wroblewski, 1984), where, $M_W$ = Natural mortality at mass W; and $W = a*L^b$, $a$ and $b$ are the regression parameters of LWR.

### 2.8. Statistical Analysis

For statistical analysis, GraphPad Prism 6.5 Software was used. The Spearman rank correlation test was applied to analyze the relationship of condition factors with TL, and BW. The one sample t-test was applied to compare the mean relative weight ($W_R$) with 100 (Anderson and Neumann, 1996). All statistical analyses were considered significant at 5% ($p$<0.05).

## 3. Results

### 3.1. Length-Frequency Distribution (LFDs)

A total 125 individuals of *N. nandus* were collected from the fishermen at different parts of the River in Rajshahi region during the present study. The LFDs showed that the smallest and largest individuals were 3.0 cm and 15.1 cm in TL, respectively; whereas the BW ranges from 0.30-52.10 g. The 7.00-8.99 cm TL size group was numerically dominant and constituted 24.0% of the total population (Figure 1).

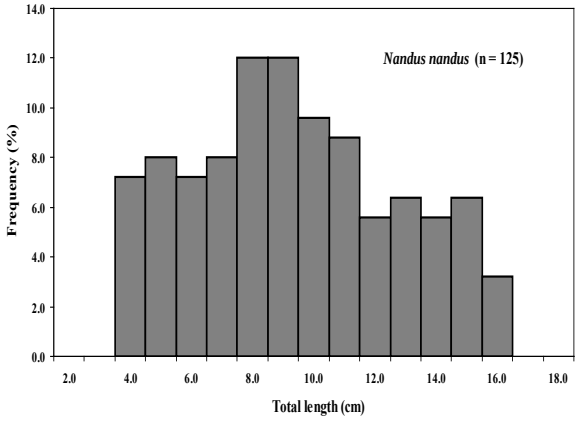


**Figure 1.** Length-frequency distribution of *Nandus nandus* from the Ganges River, northwestern Bangladesh

### 3.2. Growth Patterns

The regression parameters ($a$ and $b$) of the LWR, 95% CL of $a$ and $b$, the coefficient of determination ($r^2$) and growth pattern of *N. nandus* are given in Table 1 and in Figure 2. The $b$ value of TL *vs.* BW indicates positive allometric growth. Also, the $b$ value of LLR (TL *vs.* SL) indicates same growth pattern (positive allometric) and it is presented in Figure 2.

**Table 1.** Descriptive statistics and estimated parameters of the length-weight relationships ($W = a \times TL^b$), size at first sexual maturity ($L_m$) and form factor ($a_{3.0}$) of *Nandus nandus* (Hamilton, 1822) (n= 125) from the Ganges River, northwestern Bangladesh

| Equation | Regression parameters | | 95% CL of *a* | 95% CL of *b* | $r^2$ | GT | $L_m$ | 95% CL of $L_m$ | $a_{3.0}$ |
|---|---|---|---|---|---|---|---|---|---|
| | *a* | *b* | | | | | | | |
| $W = a \times TL^b$ | 0.0080 | 3.22 | 0.0069-0.0092 | 3.16-3.29 | 0.987 | +A | 9.1 | 7.3-11.5 | 0.0159 |
| $W = a \times SL^b$ | 0.0260 | 3.04 | 0.0230-0.0295 | 2.97-3.01 | 0.986 | I | 7.5 | 6.0-9.3 | 0.0295 |

n, sample size; *a*, intercept; *b*, slope; CL, confidence limit for mean values; $r^2$, coefficient of determination; GT, growth type; +A, positive allometric; I, Isometric growth, $L_m$, Size at first sexual maturity; $a_{3.0}$, form factor

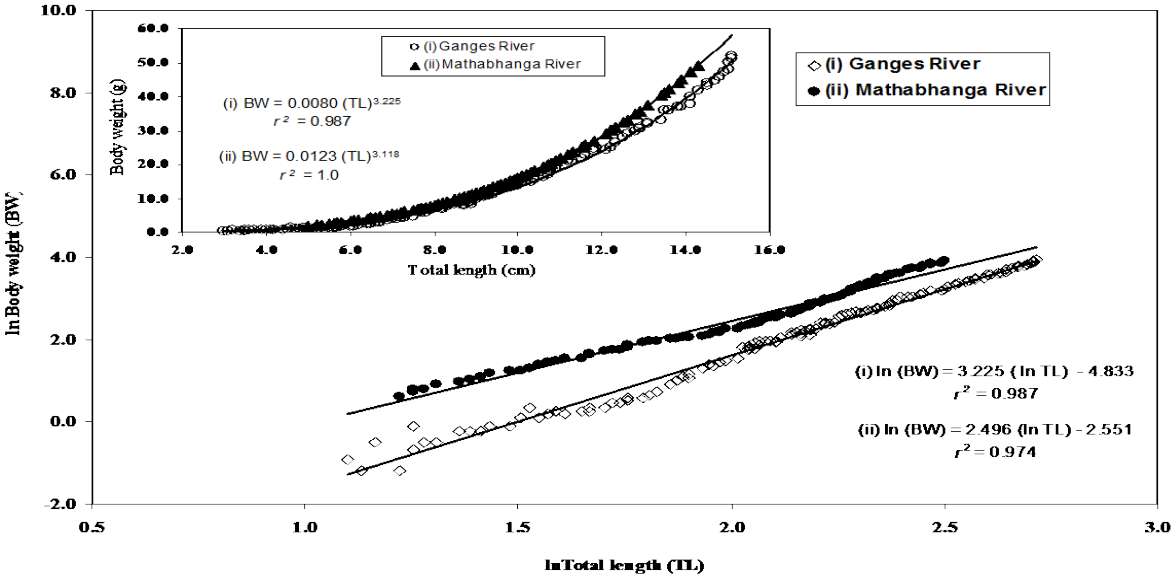


**Figure 2.** Total length (cm) and body weight (g) relationships ($W = a \times TL^b$) of *Nandus nandus* in the (i) Ganges River, NW Bangladesh (Present study), (ii) Mathabhanga River, southwestern Bangladesh (Hossain *et al*., 2006)

### *3.3. Condition Factors*

The values of all condition factors ($K_A$, $K_F$, $K_R$, and $W_R$) are given in Table 2. On the basis of Spearman rank correlation test, there were significant co-relationships of $K_F$ with TL and BW (Table 3). There was no significant different of $W_R$ from 100 ($p$=0.075) indicating a balanced population for *N. nandus* in the Ganges River (Figure 3).

**Table 2.** Condition factors; Allometric condition factor ($K_A$), Fulton′s condition factor ($K_F$), Relative condition factor ($K_R$) and Relative weight ($W_R$) of *Nandus nandus* (Hamilton, 1822) (n= 125) from the Ganges River, northwestern Bangladesh

| Condition factors | Min | Max | Mean ± SD | 95% CL |
|---|---|---|---|---|
| Allometric condition | 0.0056 | 0.0159 | 0.008± 0.001 | 0.008-0.008 |
| Fulton′s condition | 0.7633 | 2.0991 | 1.297± 0.219 | 1.258-1.336 |
| Relative condition | 0.7023 | 1.9918 | 1.018± 0.169 | 0.988-1.047 |
| Relative weight | 70.2321 | 199.1835 | 101.756± 16.850 | 98.772-104.73 |

Min, minimum; Max, maximum; SD, standard deviation; CL, confidence limit for mean values

**Table 3.** Relationships of condition factor with total length (TL) and body weight (BW) of *Nandus nandus* (Hamilton, 1822) from the Ganges River, northwestern Bangladesh

| Relationships | $r_s$ values | 95% CL of $r_s$ | *P* values | Significance |
|---|---|---|---|---|
| TL *vs.* $K_A$ | 0.0896 | -0.0926 to 0.2660 | *P* = 0.320 | *Ns* |
| TL *vs.* $K_F$ | 0.6559 | 0.5391 to -0.7480 | *P* < 0.001 | **** |
| TL *vs.* $K_R$ | 0.0889 | -0.0933 to 0.2654 | *P* = 0.324 | *ns* |
| TL *vs.* $W_R$ | 0.0890 | -0.0932 to 0.2655 | *P* = 0.324 | *ns* |
| BW *vs.* $K_A$ | 0.1101 | -0.0721 to 0.2851 | *P* = 0.221 | *ns* |
| BW *vs.* $K_F$ | 0.6751 | 0.5631 to 0.7628 | *P* < 0.001 | **** |
| BW *vs.* $K_R$ | 0.1095 | -0.0727 to 0.2846 | *P* = 0.224 | *ns* |
| BW *vs.* $W_R$ | 0.1096 | -0.0726 to 0.2847 | *P* = 0.223 | *ns* |

TL, total length; BW, body weight; $K_A$, allometric condition factor; $K_F$; Fulton′s condition factor; $K_R$, relative condition factor; $W_R$, relative weight; $r_S$, spearman rank correlation values; CL, confidence limit; *p*, shows the level of significance; *ns*, not significant; * significant; ** highly significant; **** Extremely significant

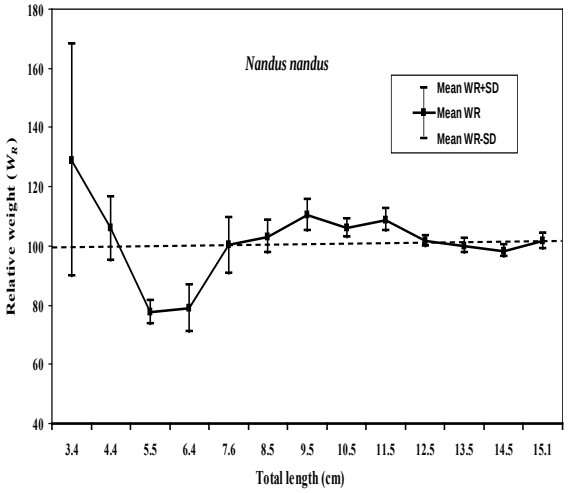


**Figure 3.** Relationships between total length (TL) and relative weight *($W_R$)* of *Nandus nandus* from the Ganges River, northwestern Bangladesh

### *3.4. Form Factor ($a_{3.0}$)*

The $a_{3.0}$ was calculated as 0.0159 for *N. nandus* in the Ganges River, NW Bangladesh. The present study also estimates the $a_{3.0}$ of *N. nandus* from world over different water bodies using available data (Table 1).

### *3.5. Size at First Sexual Maturity ($L_m$)*

The $L_m$ for the *N. nandus* was estimated as 9.10 cm TL in the Ganges River, NW Bangladesh. Moreover, the present calculates the $L_m$ of *N. nandus* from world-wide different water bodies using available studies (Table 1).

### *3.6. Natural Mortality (Mw)*

The present study revealed that $M_W$ for the population of *N. nandus* was 1.33 year$^{-1}$ in the Ganges River, NW Bangladesh and it is shown in Figure 4.

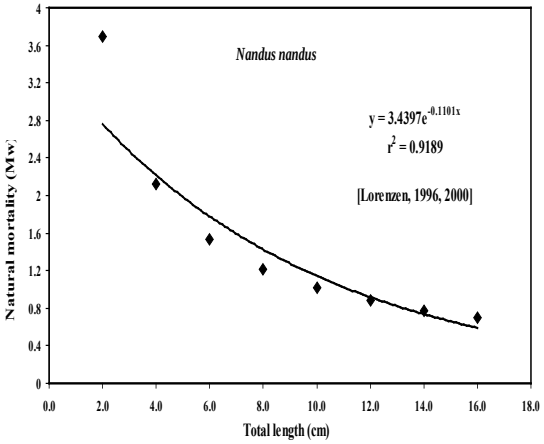


**Figure 4.** The natural mortality (Mw*)* of *Nandus nandus* from the Ganges River, northwestern Bangladesh

## 4. Discussion

Information on p opulation parameters of *N. nandus* from Bangladesh is not available, except for Hossain *et al*. (2006) from the Mathabhanga River, southwestern Bangladesh. However, the present study focuses on growth patterns, condition factors, form factor*,* reproduction and mortality of *N. nandus* from the Ganges River.

In the present study, it was not possible to sample *N. nandus* smaller than 3.0 cm in TL which may due to the absence of smaller size individual in the fishing ground or selectivity of fishing gears (Hossain *et al*., 2015d, 2016a,b,c). The maximum size was 15.1 cm in TL which is lower than the maximum recorded value of 20 c m TL (Talwar and Jhingran, 1991), but higher than 14.20 cm TL by Hossain *et al*. (2006). Information on maximum length is very significant to estimate the asymptotic length and growth coefficient of fishes, also helpful for fisheries resource planning and management (Hossain *et al*., 2012, 2016d).

In the present study, the calculated b values lies between 3.04-3.22. However, the b v alues ranging from 2.5 to 3.5 are more common (Froese, 2006). According to Tesch (1971) *b* values were close to 3, indicating isometric growth of fish and different from 3.0 indicating allometric growth (>3 positive allometric and <3 negative allometric). In the present study, the *b* values were greater than 3.0 for TL *vs.* BW and SL *vs.* BW relationships, which indicates a positive allometric growth of *N. nandus* in the Ganges River ecosystem. In addition, the *b* values (*b*=1.25) of LLR, indicate positive allometric growth. Since the present study is the first assessment on LLR, it was not possible to compare its results with other findings.

During the present study, we have worked on four condition factors ($K_A$, $K_F$, $K_R$ and $W_R$) to assess the physical and environmental condition of *N. nandus* in the Ganges River. Spearman rank correlation test expressed that the $K_F$ was significantly correlated with TL and BW. Therefore, it can be assumed that, the Fulton′s condition factor ($K_F$) is the best for determining the wellbeing of *N. nandus* in the Ganges River and adjacent ecosystem.

Wilcoxon signed rank test specify that $W_R$ was not significantly different from 100 ($p$ = 0.325) indicating the

population of *N. nandus* in the Ganges River was in balanced condition with availability of food and lower predators.

The $a_{3.0}$ was 0.0159 for *N. nandus* indicating this fish is short and deep in body shape in the Ganges River. The $a_{3.0}$ can be used to prove whether the body shape of individuals in a given population or species is extensively different from others or not (Froese, 2006).

The $L_m$ for *N. nandus* was 9.10 cm in TL. For the fishes of Bangladesh, Studies on $L_m$ are very atypical (except Hossain *et al*., 2010, 2016b). The present study offers the first effort to assess the size at sexual maturity for *N. nandus* from the Ganges River. Thus, the present study will be base for more thorough studies to find out the factors affecting the first sexual maturity and spawning size.

The $M_w$ for the population of *N. nandus* was estimated as 1.33 $year^{-1}$ in the Ganges River, NW Bangladesh. There are no earlier studies on the $M_W$ to compare with the present findings.

## 5. Conclusion

Our findings describe the population patterns of *N. nandus* including length-frequency distribution, growth pattern based on LWRs, size at sexual maturity, natural mortality, best suited condition factor, relative weight and form factor. The results of the present study would be a valuable means for fishery managers, fish biologists and conservationists to begin early management policies and regulations for the sustainable conservation of the enduring stocks of this fish species in the Ganges River and neighboring ecosystem.

## Acknowledgements

The authors would like to extend their sincere appreciation to the (i) TWAS for Research Grants (Ref: RGA No. 14-028 RG/BIO/AS_1; UNESCO FR: 324028574) for technical supports, (ii) IDRSBFRI, Bangladesh Fisheries Research Institute, Shrimp Research Station, Bagerhat, Bangladesh (Serial No. 04, Session: 2013-2014) and (iii) Ministry of Education, Bangladesh (No. 37.200000.004.003.005.2014-1309/1 (42); Date: 10-08-2014) for funding to carry out this research.

## Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of the present paper.

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