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A group of highly valuable scholars have agreed to serve on the editorial board and this places JJBS in a position of most authoritative on biological sciences. I am honored to have five associate editors: Al-Hindi (Islamic University of Gaza, Palestine.), Al-Homida, (King Saud University, Saudi Arabia), Kachani, (Western University of Health Sciences, USA), Fass, (Oman Medical College, Sultanate of Oman), and Gammoh (The University of Edinburgh). I am also delighted with our group of international advisory board members consisting from 15 countries worldwide. With our editorial board's cumulative experience in various fields of biological sciences, this journal brings a substantial representation of biological sciences in different disciplines. Without the service and dedication of our editorial board, JJBS would have never existed.

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June 2018

Prof. Khaled H. Abu-Elteen
Editor-in-Chief
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Original Articles

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The Effects of Elicited Soybean (*Glycine max*) Extract on Hematopoietic Cells of High Fat-Fructose Diet Balb/C Mice Model

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Abstract

High Fat-Fructose Diet (HFFD) triggers various metabolic problems including obesity. Obesity leads to a chronic low-grade inflammation and is associated with the unbalanced production of hematopoietic cells. In the condition of obesity, hematopoietic stem cells (HSC) are more likely to differentiate into granulocytes rather than erythrocytes as a response to inflammation. Modified soybeans are well-known to have higher compounds than the raw soybeans. One of the soybean modification processes is known as elicitation. In this study, soybeans are exposed to stressors such as *Saccharomyces cerevisiae* and light to increase the beneficial compounds inside. The elicited soybean extract (ESE) contains several anti-inflammatory and anti-oxidant compounds which are useful for the hematopoietic system. This study is aimed at determining the effects of ESE on hematopoietic cells; erythrocytes lineage (TER-119$^+$CD34$^+$, TER-119$^+$VLA-4$^+$, TER-119$^+$), and granulocytes lineage (Gr-1$^+$). In this study, twenty-eight three-week old female Balb/C mice were used. They were fed HFFD for twenty weeks, and were given ESE oral treatment for four weeks. The level of hematopoietic cells was analyzed using flow cytometry. The present study found that HFFD decreased the level of TER-119$^+$CD34$^+$, TER-119$^+$, and TER-119$^+$VLA-4$^+$ level, and increased the Gr-1$^+$ level. ESE significantly increased the TER-119$^+$CD34$^+$, TER-119$^+$ and TER-119$^+$VLA-4$^+$ level (p<0.05) and decreased the Gr-1$^+$ level (p<0.05) in the HFFD-treatment group. These results show that the potential use of ESE as an anti-inflammatory agent can improve the hematopoietic system in the HFFD-diet mice model.

Keywords: Elicited soybean extract; High fat-fructose diet; Anti-inflammatory; Hematopoiesis; Erythropoiesis, Granulopoiesis

1. Introduction

High fat consumption is known to cause over-nutrients that lead to obesity. Obesity is related to a low-grade chronic inflammation which may interfere with multiple systems in the body, above all the hematopoietic system (Benites *et al*., 2014). The hematopoietic system starts from the differentiation of hematopoietic stem cells (HSC) in the bone marrow into mature blood cells, both red and white blood cells (Hoffbrand and Moss, 2011).

Molecules CD34, TER-119 and VLA-4 are involved in erythropoiesis, while Gr-1 is involved in granulopoiesis. CD34 is a transmembrane protein which is expressed in hematopoietic progenitor cells. CD34 is also known as a specific marker for stem cells both in humans and in mice, but is not expressed in peripheral blood cells (Garland *et al*., 1997; Knowles, 2001). The TER-119 molecule is a specific marker for erythroblast and mature erythrocyte. TER-119$^+$ reacts with erythroid at different stages of development from proerythroblast to mature erythrocytes (Kina *et al*., 2000; Elliot *et al*., 2009). VLA-4 is a specific marker expressed in reticulocytes but not in mature red blood cells (Hines *et al*., 2014). Gr-1 is a specific marker for the development of granulocytes in the white blood-cell production (granulopoiesis). Gr-1 is expressed in myeloid cells through the development of granulocytes in the bone marrow (Ribechini *et al*., 2009). The expressions of Gr-1$^+$ in bone marrow determine the number of leukocytes circulating in the peripheral blood (Lee *et al*., 2011).

Obesity leads to a chronic low-grade inflammation that increases the production of pro-inflammatory cytokines, such as Tumor Necrosis Factor (TNF) and Interleukin (IL), which play a role in the HSC damage and hematopoiesis disruption (Chen *et al*., 2010; Cortez *et al*., 2013). TNF-α causes cell membrane disintegration that leads to cell death of the hematopoietic cells (Jurisic *et al*., 2011). In addition, pro-inflammatory cytokines would increase the HSC proliferation to differentiate into granulocytes in response to the inflammation and can interfere with erythropoiesis (Grigorakaki *et al*., 2011; Kraakman *et al*., 2014).

Soybeans are rich in a variety of nutrients that have several health benefits. The modification of soybeans can

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increase the nutrient contents, and/or can generate new nutrient compounds. Elicitation is one modification process through which soybeans are exposed to biotic and/or abiotic stress in order to generate secondary metabolites called glyceollin, and to increase the isoflavone content (Feng *et al*., 2007; Park *et al*., 2010; Aisyah *et al*., 2013). Glyceollin is also known as an herbal medicine for several diseases, such as cancer, hyperglycemia, and hypercholesterolemia (Park *et al*., 2012; Schexnayder and Stratford, 2015). In addition, glyceollin and isoflavone have anti-inflammatory effects which are associated with the regulation of Nitric Oxide (NO) and inducible nitric oxide synthase (iNOS) (Hämäläinen *et al*., 2007; Kim *et al*., 2011). This study investigates the effects of elicited soybean extract (ESE) on hematopoietic cells TER-119$^+$CD34$^+$, TER-119$^+$VLA-4$^+$, TER-119$^+$ and Gr-1$^+$ expression in high fat-fructose-diet (HFFD) mice.

2. Materials and Methods

2.1. Elicited Soybean Extract

In this study, the soybeans were obtained from the Indonesian Legumes and Tuber Crops Research Institute (ILETRI). The soybeans were elicited using *Saccharomyces cerevisiae* according to Kim *et al*. (2011) with several modifications (Kim *et al*., 2011). A total of one-hundred grams of soybeans was sterilized using 70 % ethanol for ten minutes, and were then rinsed with sterile distilled water four times. The sterilized soybeans were then soaked in sterile distilled water for twenty-four hours at the room temperature. After twenty-four hours of soaking, the soybeans were grown on cotton media moistened with distilled water. Every one-hundred grams of soybens were inoculated with 7.5 mL *Saccharomyces cerevisiae* with a concentration of 10^7. After that, the soybeans were covered with a plastic wrap and were put under a light bulb for sixteen hours per day for three days (according to Aisyah *et al*. (2013) (with slight modification). The extraction was performed using 300 mL of 80% ethanol for every one-hundred grams of soybeans. In the next step, the ethanol was evaporated from the extract. The extract was then frozed dried to obtain the paste extract of ESE.

2.2. Animals

This research used twenty-eight three-week old female mice of broodstock obtained from The Integrated Research and Testing Laboratory, Gajah Mada University. The animals were grouped into seven groups containing four mice in each. The normal-diet (ND) group (K1), a normal diet group which were treated with ESE dose of 104 mg/kg BW (P1), and the high fat-fructose diet (HFFD) group (K2) served as a control in this study. We also used an anti-cholesterol drug Simvastatin dose of 2.8 mg/kg BW (P2) as a comparison (Mahmoud *et al.*, 2013). The ESE treatment doses in this study were 78 mg/kg BW (P3), 104 mg/kg BW (P4), and 130 mg/kg BW (P5). All protocols in this study were approved by University of Brawijaya Ethics Committee (Reg. No. 647-KEP-UB).

2.3. High Fat-Fructose Diet (HFFD) and Elicited Soybean Extract (ESE) Treatment

The composition of the high fat-fructose food consisted of 30% fructose, 10% carbohydrate, 35% Hi Gro551 (starter pellets for pigs contain of 73.9% carbohidrate, 20.5% protein, 5% fat, 1.6% fiber, vitamins and minerals by Charoen Pokphand Company, Indonesia), 17% cow fat, and 8% yolk cholesterol. The three-week old female mice were given HFFD diet for twenty weeks, and were then given ESE treatment of the following doses; 78 mg/kg BW, 104 mg/kg BW, and 130 mg/kg BW; and Simvastatin dose 2,8 mg/kg BW for four weeks by oral administration.

2.4. Bone Marrow Isolation

Mice were dissected after four weeks of treatment, then both femurs of each mouse were isolated. The bone-marrow isolation procedures were based on Khasanah *et al*. (2015) protocol. Bone marrow cells were isolated by cutting both ends of the femur and were then flushed out using 1 mL of phosphate buffered saline (PBS) in 1 mL syringe. The flushed-cells were then collected into centrifuge tubes, adding 10 mL of PBS, and were then centrifuged at 4500 rpm, 10°C, for five minutes. Pellet was resuspended in 1 mL of PBS for antibody staining procedures.

2.5. Immunostaining

Antibody staining in this study included fluorescein isothiocyanate (FITC)-conjugated rat anti-mouse CD34, FITC-conjugated rat anti-mouse Gr-1, Phycoerythrin (PE)-conjugated rat anti-mouse VLA-4, and PE-conjugated rat anti-mouse TER-119. A mixture of 250 µL of cell suspension and 350 µL of PBS were centrifuged at 4500 rpm, 10°C for five minutes. Pellet was resuspended in 50 µL PBS and specific antibodies, and was then incubated in the ice box for twenty minutes. Flow cytometry analysis was performed to determine the expression of TER-119$^+$CD34$^+$, TER-119$^+$VLA-4$^+$, TER-119$^+$ and Gr-1$^+$ cells.

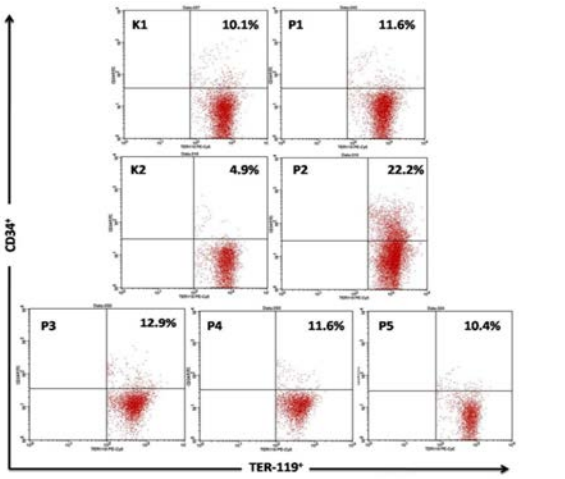
2.6. Data Analysis

Data were analyzed by the BD Cell Quest PRO™ software and were then analyzed statistically using SPSS (version 16.0) for Windows using one-way ANOVA. Significant data were further tested using Duncan test.

3. Results

3.1. Elicited Soybean Extract (ESE) on Red Blood Cells Progenitor TER-119+CD34+

The level of progenitor cells TER-119$^+$CD34$^+$ significantly decreased after the HFFD administration for twenty-four weeks (4.9%) ($p < 0.05$) compared to normal (10.1%) (Figure 1). All ESE doses increased the level of TER-119$^+$CD34$^+$ (12.9%, 11.6%, 10.4%, respectively) and the highest number of TER-119$^+$CD34$^+$ cells was found in the HFFD-mice treated with Simvastatin (Figure 1). Moreover, the ESE of all doses and the Simvastatin administration normalize the relative number of TER-119$^+$CD34$^+$ cells.



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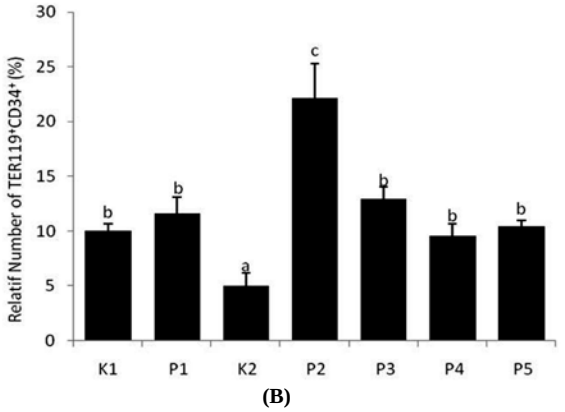


(B)

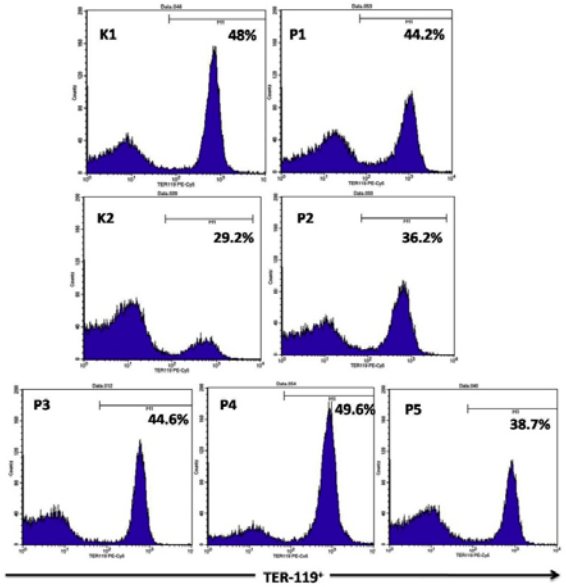
Figure 1. Elicited soybean extract (ESE) improved the level of red blood-cell progenitor TER-119$^+$CD34$^+$ in the bone marrow. K1: normal diet; P1: normal diet + ESE 104 mg/kg BW; K2: high fat-fructose diet (HFFD); P2: HFFD + Simvastatin 2,8 mg/kg BW; P3: HFFD + ESE 78 mg/kg BW; P4: HFFD + ESE 104 mg/kg BW; P5: HFFD diet + ESE 130 mg/kg BW (A) Flowcytometry analysis of TER-119$^+$CD34$^+$ expression in bone marrow cells. All ESE doses and Simvastatin increased the level of TER-119$^+$CD34$^+$ (B) TER-119$^+$CD34$^+$ expression in each group analyzed by One-way ANOVA. ESE improved the hematopoietic system by increasing the level of TER-119$^+$CD34$^+$ in all doses compared to the HFFD control. Letters above each bar indicate a significant difference.

3.2. Elicited Soybean Extract on Erythropoiesis

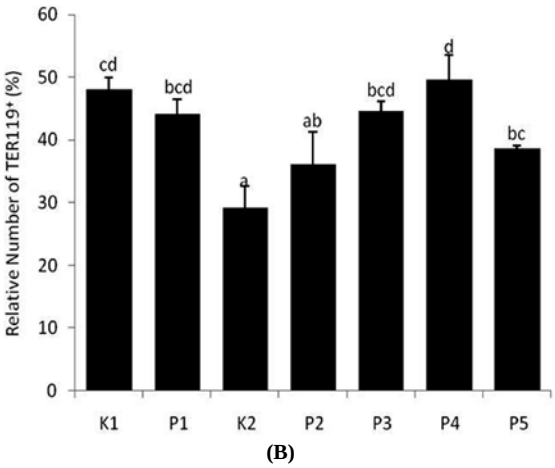
This study also observed the expression of erythroid TER-119$^+$ and reticulocytes TER-119$^+$VLA-4$^+$ in bone marrow cells. The HFFD administration for twenty-four weeks decreased the level of erythroid TER-119$^+$ (29.2%) compared to the normal group (48%) ($p < 0.05$). The administration of ESE from low to high doses was able to improve erythropoiesis by increasing the level of erythroid TER-119$^+$ significantly in the HFFD group ($p<0.05$) (44.6%, 49.6 %, 38.7 %, respectively) (Figure 2).

Moreover, the TER-119$^+$VLA-4$^+$ level is also decreased in the HFFD group (40.6 %) compared to the normal group (63.9 %) ($p < 0.05$) (Figure 3). The ESE dose of 104 mg/kg BW which was given to the normal group increased the level of TER-119$^+$VLA-4$^+$ (54.1 %) but with no significant difference from normal and HFFD groups. The HFFD mice were given ESE doses of 104 mg/kg BW and 130 mg/kg BW which significantly increased the expression of TER-119$^+$VLA-4$^+$ (79.8 %, 70.2 %, respectively) ($p<0.05$), while the 78 mg/kg BW increased the level of TER-119$^+$VLA-4$^+$ approaching the normal group (50%). The HFFD mice that were given 2,8 mg/kg BW of Simvastatin also had the level of TER-119$^+$VLA-4$^+$ increased in the HFFD group (79.4%) ($p<0.05$) (Figure 3).

In the erythroid lineage, all doses of ESE were capable to normalize the level of hematopoietic cells of the HFFD mice from the early stage up to the late stage. In the early stage of erythroid development, ESE was capable to normalize the level of progenitor red blood cells. ESE also normalized the level of red blood-cell precursors in the late development of erythroid lineage.



(A)



(B)

Figure 2. The level of erythroid cells TER-119$^+$ increased after ESE treatment. K1: normal diet; P1: normal diet + ESE 104 mg/kg BW; K2: high fat-fructose diet (HFFD); P2: HFFD + Simvastatin 2,8 mg/kg BW; P3: HFFD + ESE 78 mg/kg BW; P4: HFFD + ESE 104 mg/kg BW; P5: HFFD diet + ESE 130 mg/kg BW (A) TER-119$^+$ expression in bone marrow cells. All doses of ESE and Simvastatin increased the level of TER-119$^+$ in HFFD mice (B) TER-119$^+$ expression comparison between each group analyzed by One-way ANOVA. All doses of ESE improved the level of erythroid TER-119$^+$ in the HFFD-fed mice. Letters above each bar indicate a significant difference.

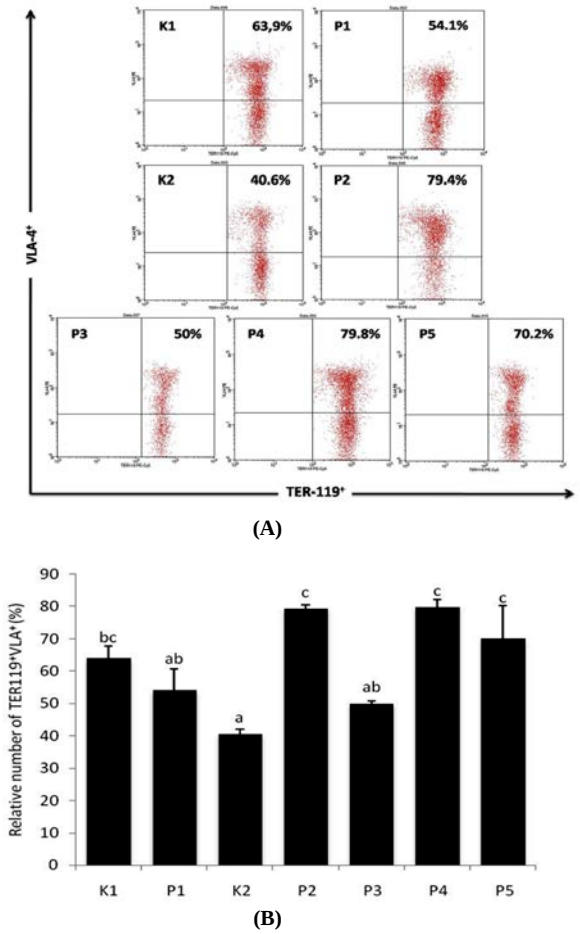


Figure 3. The level of reticulocyte cells TER-119$^+$VLA-4$^+$ increased after ESE treatment. K1: normal diet; P1: normal diet + ESE 104 mg/kg BW; K2: high fat-fructose diet (HFFD); P2: HFFD + Simvastatin 2,8 mg/kg BW; P3: HFFD + ESE 78 mg/kg BW; P4: HFFD + ESE 104 mg/kg BW; P5: HFFD diet + ESE 130 mg/kg BW (A) Flowcytometry analysis of TER-119$^+$VLA-4$^+$ expression in bone marrow cells. All doses of ESE and Simvastatin increased the level of TER-119$^+$ VLA-4$^+$ in the HFFD-fed mice (B) The bars show the expression of TER-119$^+$VLA-4$^+$ in each group. Simvastatin and all doses of ESE improved the level of erythroid TER-119$^+$VLA-4$^+$ in the HFFD-fed mice. Letters above each bar indicate a significant difference.

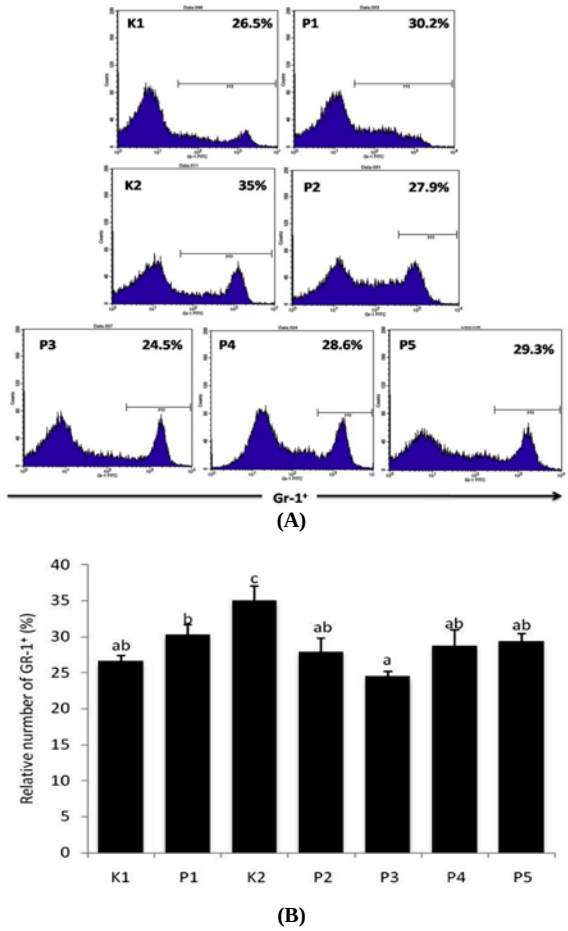


Figure 4. Elicited soybean extract (ESE) treatment decreased the level of granulocyte precursors Gr-1$^+$ in bone marrow cells. K1: normal diet; P1: normal diet + ESE 104 mg/kg BW; K2: high fat-fructose diet (HFFD); P2: HFFD + Simvastatin 2,8 mg/kg BW; P3: HFFD + ESE 78 mg/kg BW; P4: HFFD + ESE 104 mg/kg BW; P5: HFFD diet + ESE 130 mg/kg BW (A) Gr-1$^+$ expression in bone marrow cells. All doses of ESE and Simvastatin decreased the level of Gr-1$^+$ in the HFFD-fed mice (B) The bars show the expression of Gr-1$^+$ between each group analyzed by One-way ANOVA. Simvastatin and all doses of ESE decreased the level of granulocytes precursors Gr-1$^+$ in the HFFD-fed mice. Letters above each bar indicate a significant difference.

3.3. Elicited Soybean Extract on Granulopoiesis

HFFD feeding increased the level of Gr-1$^+$ (35%) compared to the normal group (26.5%) (p<0.05). ESE in all doses suppressed the expression of Gr-1$^+$ in bone marrow (p<0.05), (24.5%, 28.6%, and 29.3%, respectively). Similar to the ESE results, the administration of Simvastatin dose of 2,8 mg/kg BW also decreased the level of Gr-1$^+$ until close to the normal group level (27.9%) (p<0.05) (Figure 4).

4. Discussion

Nowadays, obesity is a common health problem. The prevelence of obesity involves adults and children and its rates are increasing in most countries arround the world. The lack of physical activity, as well as the high fat-sugar food consumption may increase the occurrence of obesity (Antipatis and Gill, 2001). On the other hand, the consumption of foods with rich nutrient content can reduce the risk of obesity (Troesch *et al*., 2015).

Soybeans contain rich nutrients both in raw, as well as in modified forms. The elicitation of soybeans can increase the isoflavone contents, such as genistein and daidzein and can also produce glyceollin, a new secondary metabolite compound (Feng *et al*., 2007; Park *et al*., 2010; Aisyah *et al*., 2013).

This study shows that the HFFD administration leads to obesity (data not shown) and may be associated with an inflammation that affects the hematopoietic system. The

HFFD-fed mice have impaired red blood-cell development by decreasing the level of red blood-cells progenitors (TER-119$^+$CD34$^+$) and (TER-119$^+$ and TER-119$^+$VLA-4$^+$). The impairment in the hematopoietic system may be related to an inflammation that leads to erythropoiesis impairment and interferes with iron absorption which disrupts the erythroid maturation in red blood-cell production (Ganz, 2005; Martins, 2014). A previous study has shown that the inflammation decreased the regulator complement of red blood-cells, resulting in the lysis of red blood-cells progenitor (Safitri *et al.,* 2017). The inflammation was also marked by cytokines pro-inflammatory production such as TNF-α, IL-1β, and IL-6 (Kraakman *et al*., 2014). One study related to hematopoietic cells showed that after TNF-α treatment, cell membrane transduced many signals for apoptosis and necrosis (Jurisic *et al*., 2011). Another study indicated that TNF-α inhibits CD34$^+$ hematopoietic progenitor cells proliferation (Rusten *et al*., 1994).

The ESE treatment for four weeks improved the red blood-cells development in the HFFD-fed mice. Every ESE dose normalized the level of TER-119$^+$CD34$^+$ progenitors cells in the HFFD-fed mice. These results are supported by previous studies which stated that genistein has a role in regulating hematopoietic progenitor cells to reduce the risk of DNA damage in the bone marrow cells (Day *et al*., 2013). The expression of red blood-cell precursors, TER-119$^+$ and TER-119$^+$VLA-4$^+$ were normalized as well after four weeks of the ESE treatments. Similar to the ESE treatment, the simvastatin increased the TER-119$^+$CD34$^+$ progenitor cells to a level higher than all doses of ESE treatment, but the expression of TER-119$^+$ and TER-119$^+$VLA-4$^+$ cells have no significant difference compared to the ESE treatment. Simvastatin is well known to have a potential effect on the hematopoiesis improvement through the ability as an anti-inflammatory agent. Previous studies showed that simvastatin has a role as an anti-inflammatory agent, increasing the HSC number through the bone marrow improvement, and increasing erythropoiesis by targeting regulatory pathways of hepcidin and iron (Lefer, 2002; Chang *et al*., 2011; Bajaj *et al* 2015).

Our finding revealed that the effectiveness of the ESE is not only relevent to the red blood- cell early development, but also in the late development. This is indicated by the fact that ESE is able to normalize not only the level of progenitor cells but also the precursors which were decreased after the HFFD treatment. Glyceollin and daidzein in ESE have the potential effects of reducing ferric ion (Fe^{3+}) to be a ferrous ion (Fe^{2+}), thus making it easier to be absorbed (Kim *et al*., 2010). Iron absorption is an important factor in hemoglobin synthesis (Hoffbrand and Moss, 2011). Vitamin C in the soybean improves the iron absorption for hemoglobin synthesis during erythropoiesis and is able to prevent the formation of insoluble and unabsorbable iron (Hallberg *et al*., 1989). However, calcium which is also found in the soybean has a contrastive effect on hematopoiesis. Calcium inhibited the iron absorption for the hemoglobin synthesis by competing with iron to bind with same substrates which have an important role in the iron absorption process (Hallberg *et al*., 1991). This condition may explain why the potential dose of ESE in each parameter is different.

Obesity induces Granulocyte-Colony Stimulating Factor (G-CSF) to stimulate the production of granulocytes and further increase the recruitment of macrophages by adipose cells (Benites *et al*., 2014). Obesity increases Hematopoietic Stem Cell (HSC) differentiation into monocytes (Singer *et al*., 2014). HSC proliferation and differentiation in the bone marrow occur continuously in response to inflammation and decrease the ability to repair themselves (self-renewal) eventually causing imbalanced blood-cell production (Schuettpelz and Link, 2013). This study indicated that the level of Gr-1$^+$ increased significantly in the bone marrow cells of the HFFD-fed mice. Gr-1 is a specific marker for granulocytes in white blood-cell production (granulopoiesis). After the ESE treatment, Gr-1$^+$ level decreased significantly approaching the normal value. This finding can be attributed to the ability of genistein as anti-inflammatory (Kim *et al*., 2011) which reduces the ability of G-CSF to induce the proliferation of HSC occuring continuously due to the inflammation, but does not reduce the ability of G-CSF in accelerating the granulocytes differentiation (Zhou *et al*., 2005; Day *et al*., 2013; Souza *et al*., 2014).

The current study speculated that the ESE contents, such as glyceollin, genistein, daidzein, vitamins among others related to each other in improving the hematopoietic system. Based on the previous explanation, ESE can be used as an alternative medicine because of the effectiveness of its herbal ingredients as anti-inflammatory. Moreover, ESE is more likely to have fewer side effects than synthetic medicines.

5. Conclusion

Elicited soybean extract (ESE) improves the development of hematopoietic cells by increasing the level of TER-119$^+$CD34$^+$, TER-119$^+$, and TER-119$^+$VLA-4$^+$ as well as lowering the level of Gr-1$^+$ in the bone marrow cells of HFFD-fed mice. ESE can be an effecctive anti-inflammatory agent due to its ability to improve the hematopoietic system of obese mice.

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Observations on the Morphometric and Meristic Characters of Guinean Tilapia, *Coptodon guineensis* (Günther, 1892) (Family: Cichlidae) from the Buguma Creek and the New Calabar River in Nigeria

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Abstract

This research was conducted to study the morphological characteristics of *Coptodon guineensis* from brackish and fresh water habitats in Rivers State, Nigeria using morphometric measurements and meristic counts. A total of 200 specimens of *Coptodon guineensis*, (two groups of 100 each from two study sites) were examined and analyzed for morphometric differentiation. The results showed that almost all the values of the external morphometric parameters were higher in the New Calabar River population than those of the population from Buguma Creek. There were significant difference (p<0.05) in the weight, total length, standard length, pre anal fin length, pre pelvic fin length, penduncle depth and body depth. The mean percentage standard length to PrEOL of C. *guineensis* from Buguma Creek (10.54±0.29 %) was significantly higher p<0.05 than that from the New Calabar River (9.65±0.27 %) and the mean percentage SL to BD of C. *guineensis* from the New Calabar River (91.05±2.26 %) was significantly higher (p<0.05) than that from the Buguma Creek (84.56±1.54 %). All the external parameters displayed allometric growth expect for PoOL and BD. The morphometric relationships between SL vs PrAFL showed high coefficients of determination (>0.88) and moderate coefficient (>0.56) with PD in the population of the New Calabar River, while the population of the Buguma Creek PD showed high coefficients of determination (>0.62). The specimens investigated in this study reveal that the freshwater population could be phenotypically separable from the brackish water population.

Keywords: *Coptodon guineensis*, Guinean tilapia morphological characteristics, Buguma Creek, New Calabar River,

1. Introduction

Historically, the morphology of fishes has been the primary source of information for taxonomic and evolutionary studies. Under the basic concept of evolution, every species is believed to be undergoing micro and macro evolutionary process resulting in the expression of significant genetic variations at the levels of the species specific chromosome morphology/structure, gene controlled protein structure and polygene controlled morphometrics and metrics (Ayala and Keiger 1980). Also, as a rule, specimens originating from different areas differ from one another in morphology (Franičevič *et al*., 2005). The shape and structures are unique to the species, and the variation in its feature is probably related to the habit and habitat among the variants of this s pecies (Cavalcanti *et al*., 1999), which is determined by the evolutionary background of the fish and the physical and chemical characteristics of water.

Information on the sub-stock structure in fish populations is essential for the management of many stocks. Muzinic and Marr (1960) have stated the biological and fishery management principle that necessitates the racial/stock/sub-species differentiation of fishery resources as "the logical and practical reasons for identifying population units are that such units may have their own characteristics of recruitment, growth, natural mortality, migration, behavior etc. more or less independent of the characteristics of other population units within the same species." Grant *et al*., (1980) pointed out the importance of delineating the stocks and their boundaries which have become an essential part of fishery management/ conservation. For effective fishery management and implementation of the worthwhile stock rebuilding programs, knowledge of the stock structure, distribution of the fishing effort and mortality amongst the various components are essential, since each stock must be managed separately to optimize their yield (Grimes *et al*., 1987; Carvalho and Hauser 1994 ; Begg *et al*., 1999).

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There are many approaches that can be taken to determine the stock or population structure of any fish species including genetic analyses, phenotype analyses to detail the growth rates, age composition, morphometrics and micro constituents in calcified structures, together with parasite loads and tagging returns. Morphometric measurements and meristic counts are considered authentic and the easiest methods for the identification of specimen termed as morphological systematics (Nayman 1965). The morphometric analysis helps to understand the relation between body parts (Carpenter *et al*., 1996) and to know the origin of stock, separation of stocks, or identification of the commercially-important species of fishes (Devi *et al*., 1991; Kohinoor *et al*., 1995; Narejo *et al*., 2000).

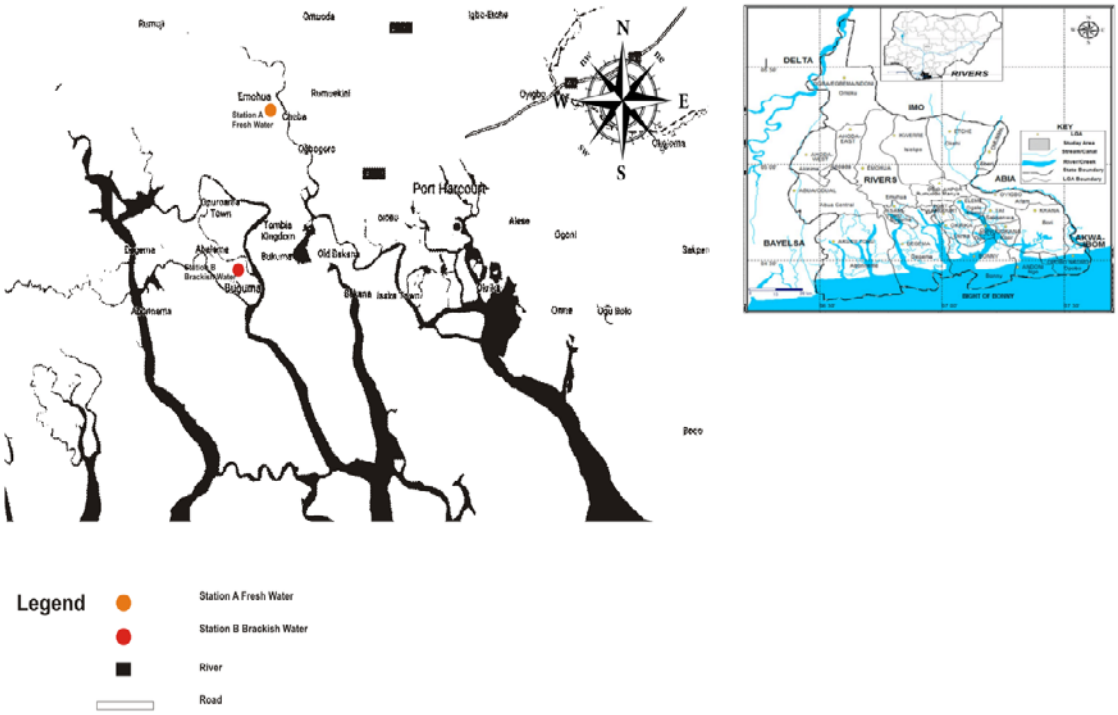
The family Cichlidae (Cichlids) represents the most species rich-family of vertebrates (Kocher 2004). The cichlids *Coptodon guineensis*, is a brackish water euryhaline species found along the West coast of Africa (Philippart and Ruwet 1982). Formerly included in *Tilapia*, in 2013 this genus was separated by (Dunz and Schliewen 2013). This species was formerly and wrongly referred to as *Tilapia melanopleura* in many parts around the world (Philippart and Ruwet 1982). The species, popularly-called *Tilapia guineensis*, can be easily separated from other Tilapia species occurring in Nigerian freshwaters because of its emarginated caudal fin and the prolongation of the rayed part of the dorsal fin (Adesulu and Sydenham 2007). The species also adapted to diverse habitat permanent and temporary rivers, opened and closed estuarine, lagoon, swampy lakes, deep lakes and coastal brackish lakes (Trewavas 1983). Cichlids exhibit remarkably high levels of genetic and morphological diversity, which affects their morphology, ecology, behaviour and genomes (Nelson 1994; Barlow 2000; Chakrabarty 2005).

In Nigeria, systematic studies are lacking, despite their great significance for a better management of the stocks. There are very few documented studies on population/stock structure in fishes using morphological traits in Nigeria. The objective of this study is to investigate the morphological variations in *Coptodon guineensis* from the New Calabar River and the Buguma Creek, and to prepare accurate guides for the identification of species in Nigeria.

2. Materials and Methods

2.1. Study Area

The samplings were conducted at the New Calabar River and Buguma Creek in Rivers State, Nigeria (Figure 1). The New Calabar River is situated about 15 km from Port Harcourt City, located in Obio - Akpo Local Government Area of Rivers State between longitude 6.8985°E and latitude 4.8888°N of the Greenwich meridian. The New Calabar River is characterized by fresh water. The Buguma Creek is located southeast of Niger Delta between Longitude 6°47°E and 6°59°E and Latitude 4°31°N and 4°59°N in Asari - Ton Local Government Area Rivers State. The Buguma Creek system consists of a main creek channel and some associated interconnecting creeks, lying along the coast of Nigeria. The Buguma Creek is characterized by brackish water.



Map showing the sampling site.

C. guineensis were identified using the keys and works of (Adesaulu and Sydenham 2007). One hundred specimens of *C. guineensis* were randomly selected from the local fishermen catches using cast net, gillnet and beach seine at the two locations and were then taken to the laboratory. The samples of *C. guineensis,* ranging between 120 mm and 150 mm standard length, were collected monthly from both sites over the period from February to July of 2017. The specimens were transported in ice chests to the laboratory, where measurements started immediately to avoid shrinkage. Various equipment including Calipers, a pair of divider, and a graduated meter rule were used in the measurements. Morphometric measurements were taken according to the descriptions given in Gupta and Gupta (2006). All fish were measured for total length (TL) and standard length (SL) to the nearest 0.1 cm, and were weighed (body weight, BW) to the nearest 0.1. The following morphometric data were taken from the left side of the fish body. These were: Standard length (SL), Total length (TL), Weight (W), Dorsal fin length (DFL), Head length (HL), Peduncle Length (PL), Peduncle depth (PD), Body depth (BD), Eye diameter (ED), Pre dorsal fin length (PrDFL), Pre anal fin length (PrAFL), Pre pelvic fin length (PrPFL), Caudual fin length (CFL), Pre orbital length (PrOL) and Post orbital length (PoOL). Seven meristic characters were also investigated with the aid of a magnifying glass. These were: Dorsal fin ray (DFR), Anal fin ray (AFR), Pectoral fin ray (PFR), Pelvic fin ray (PVFR), Scales along lateral line (SALL), Dorsal fin spine (DFS) and Anal fin spine (AFS)**.**

In order to standardize the differences in the overall body size among the specimens, all the morphometric measurement data were divided by standard length (SL) and were presented as ratio (Hubbs and Lagler 1947). Basic descriptive statistics (minimum, maximum, mean, and standard deviation) were calculated for the morphometric measurements and meristic counts. A t-test was applied to determine the significant differences in the two populations.

For the purpose of growth variability of all the external morphometric characters studied with respect to standard length, linear regression analysis was carried out and the strength of the relationship was determined using the r^2 value. While *p*-value was used to determine the significance of the relationship. Morphometric characters were adjusted to size by running log-log regressions between SL and each character (Reist 1985). The Analysis of Covariance (ANCOVA) Zar (1984) was used to test for significant differences in slopes and intercepts among the relationships.

3. Results

A total of 200 specimens of *C. guineensis* (two groups of 100 each) from two study sites were examined and analyzed. Table 1 shows that almost all the values of the external morphometric parameters were higher in the New Calabar River population than those of the population from the Buguma Creek. For example, the total length and total weight of *C. guineensis* from the New Calabar River (17.06 ± 0.44cm and 124.55 ± 10.34g respectively) were higher than those of the Buguma Creek (14.75 ± 0.29 cm and 81.12 ± 6.730g respectively). Similar results were recorded for all of the morphometric measurements, apart from the eye diameter and penduncle length (1.18 ± 0.02cm and 1.76 ± 0.05cm respectively), that were slightly higher in the population of *C. guineensis* from Buguma Creek than those from the New Calabar River (1.17 ± 0.02cm and 1.65 ± 0.05cm respectively). However, the pre-orbital length was constant with a mean value of 2.20±0.03 recorded for the New Calabar River population and 2.20±0.04 in the population from the Buguma Creek (Figure 1). The statistical analysis of the morphometric parameters as shown in Table 1, indicates that there were significant differences ($p<0.05$) in the weight, total length, standard length, pre- anal fin length, pre-pelvic fin length, penduncle depth and body depth along with other features as the eye diameter, head length, pre-orbital length, post-orbital length and penduncle length which showed that the fish, in all probability, were obtained from two statistically-indistinguishable races or stocks.

Analyses of the meristic characters revealed variation in the dorsal fin ray with the New Calabar River population having 13-14 and 10-13 for the Buguma Creek (Table 2). Also, the New Calabar dorsal fin spine range (14-17) was slightly more than that of the Buguma Creek range (15-16). The results further showed a significant difference in the number of anal fin ray ($p< 0.05$) between the two populations. The pelvic fin rays, pelvic spines and anal fin spines remained constant between the two populations.

Table 1. Mean and standard error for morphometric of *Coptodon guineensis* from the Buguma Creek and the New Calabar River.

Traits	New Calabar River		Buguma Creek		F	t	*p*-value
	Range	Mean ± SE	Range	Mean ± SE			
Weight	23 – 640	124.55 ± 10.34 [a]	17 – 520	81.12 ± 6.73 [b]	11.449	3.522	0.00
Total length	3.5 - 29.8	17.06 ± 0.44 [a]	1.5 - 23.5	14.75 ± 0.29 [b]	10.371	4.335	0.00
Standard length	6.2 - 24.9	13.45 ± 0.38 [a]	5.7 - 19.5	11.27 ± 0.23 [b]	23.39	4.86	0.00
Pre orbital length	1 - 3.1	1.22 ± 0.03	0.4 - 2.1	1.16 ± 0.03	2.795	1.552	0.12
Post orbital length	1.1 - 2.7	2.20 ± 0.03	1.2 - 3.5	2.20 ± 0.04	9.761	0.068	0.95
Head length	2.1 - 5.3	3.43 ± 0.04	2.2 - 4.5	3.37 ± 0.04	0.846	1.045	0.30
Eye diameter	1 – 2	1.17 ± 0.02	0.9 - 2.2	1.18 ± 0.02	0.413	-0.557	0.58
Pre-dorsal fin length	2.4 - 5.6	3.73 ± 0.04	2.6 - 4.8	3.69 ± 0.04	1.178	0.637	0.53
Pre anal fin length	2.1 - 21.8	9.22 ± 0.39 [a]	4.2 – 15	7.16 ± 0.23 [b]	22.854	4.557	0.00
Pre pelvic fin length	2.6 - 5.8	3.88 ± 0.04 [a]	2.8 - 4.9	3.75 ± 0.04 [b]	3.865	2.112	0.04
Caudual fin length	3 - 4.9	3.89 ± 0.04 [a]	3 – 4.4	2.24 ± 0.08 [b]	35.911	17.926	0.00
Peduncle length	0.8 - 3.4	1.65 ± 0.05	1 – 3.4	1.76 ± 0.05	0.02	-1.617	0.11
Peduncle depth	1.1 - 3.9	2.26 ± 0.08 [a]	1 – 3.4	1.83 ± 0.04 [b]	40.543	4.716	0.00
Body depth	5.6 - 23.8	11.87 ± 0.39 [a]	3.2 - 13.4	9.36 ± 0.17 [b]	9.756	5.957	0.00

[abc]Mean (± Standard error) in the same column having similar superscript are not significantly different (p>0.05). F = Levene's Test for Equality of Variances, t= t- Test.

Table 2. Mean and standard error for meristic of *Coptodon guineensis* from the Buguma Creek and the New Calabar River.

Traits	New Calabar River		Buguma Creek		F	t	*p*-value
	Range	Mean ± SE	Range	Mean ± SE			Mean ± SE
Dorsal fin ray	13 - 14	12.05 ± 0.14 [b]	10 – 13	12.64 ± 0.07 [a]	19.366	-3.685	0.00
Dorsal fin spine	14 - 17	15.57 ± 0.06	15 – 16	15.64 ± 0.05	8.03	-0.903	0.37
Scale along the lateral line	19 - 25	21.82 ± 0.10	19 – 23	21.87 ± 0.29	2.021	-0.161	0.87
Anal fin ray	9 -10	9.84 ± 0.04 [a]	8 – 10	9.12 ± 0.03 [b]	2.667	14.623	0.00
Pelvic fin ray	5	5.00 ± 0.00	5	5.00 ± 0.00	-	-	-
Anal fin spine	3	3.00 ± 0.00	3	3.00 ± 0.00	-	-	-
Pelvic fin spine	1	1.00 ± 0.00	1	1.00 ± 0.00	-	-	-

[abc]Mean (± Standard error) in the same column having similar superscript are not significantly different (p>0.05).

F = Levene's Test for Equality of Variances, t= t Test.

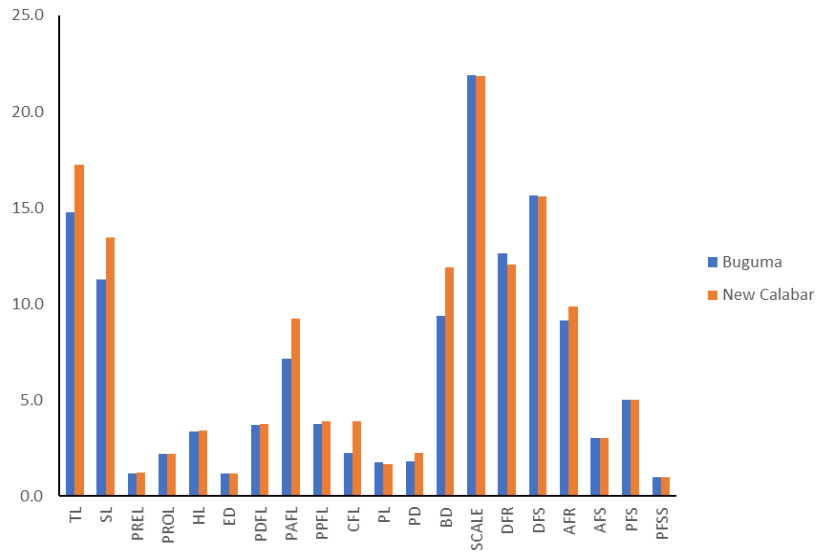


Figure 1. Comparison between the morphometric characters of the *Coptodon guineensis* in the Buguma Creek and the New Calabar River.

The percentages of morphometric characters expressed as the percentage of standard length, % SL, morphometric (Table 3) , were found to be significantly different between the two populations (p<0.05). The mean percentage standard length to PrEOL of *C. guineensis* from the Buguma Creek (10.54±0.29 %) was significantly higher p<0.05 than that from the New Calabar River (9.65±0.27 %), and the mean percentage BD to SL of *C. guineensis* from the New Calabar River (91.05±2.26%) was significantly higher (p<0.05) than that from the Buguma Creek (84.56±1.54%). However, eight percentage morphometric variables in relation to the standard length revealed highly significant differences between the two populations (p<0.01). The spcimens investigated in this study revealed some discrimination indicating that the freshwater population could be separable from the brackish water population.

The comparative study of the meristic counts with the standard length revealed clearer discriminating differences between the specimens of the two populations than the morphometric measurements (Table. 4). There were significant differences (p < 0.01) in the mean percentage standard lenght of all the meristic traits, except in the mean percentage standard length of AFR for which there were significant differences between the two populations (p < 0.05). Only PD showed no significant difference between the two populations in the percentage of morphometric variables in relation to the standard length.

In this study, eleven characters were regressed against the standard length, and several correlations were observed as in Table 5. Examination of *C guineensis* from the New Calabar River indicated that the allometric growth is relative to the standard length in most of the characters considered apart from the PrOL, PL, ED and PD with isometric growth pattern (b=3). Further results of the external morphometric characteristics' growth variability of the *C guineensis* from the Buguma Creek studied with respect to SL (Table 5), showed that all the external parameters displayed allometric growth expect for PoOL and BD. The morphometric relationships between SL vs PrAFL showed high coefficients of determination (>0.88) and moderate coefficient (>0.56) with PD in the population from the New Calabar River, while the population from the Buguma Creek PD showed high coefficients of determination (>0.62). The remaining characters had a very low level of relationship (Table 5). In the four meristic characters analyzed, all did not express any relativeness to the standard length. The correlation coefficient (r) values recorded in both populations were very low (Table 6).

Table 3. Morphometric characters of *C. guineensis* expressed as percentage of Standard length from the Buguma Creek and the New Calabar River.

Morphometric Traits	New Calabar River	Buguma Creek	F	t	*p*-value
Pre orbital length	9.65±0.27	10.54±0.29*	0.512	-2.232	0.03
Post orbital length	17.64±0.50	20.29±0.53**	0.002	-3.62	0.00
Head length	27.32±0.73	30.98±0.64**	1.514	-3.757	0.00
Eye diameter	9.24±0.24	10.93±0.28**	2.441	-4.562	0.00
Pre dorsal fin length	29.71±0.79	33.99±0.71**	1.505	-4.032	0.00
Pre anal fin length	66.55±1.15**	62.48±0.81	6.681	2.896	0.00
Pre pelvic fin length	30.91±0.83	34.66±0.84**	0.223	-3.184	0.00
Caudual fin length	30.89±0.83**	20.54±0.81	0.269	8.912	0.00
Peduncle length	12.46±0.28	15.65±0.34**	0.392	-7.159	0.00
Peduncle depth	16.87±0.38	16.26±0.23	9.621	1.356	0.18
Body depth	91.05±2.26*	84.56±1.54	21.116	2.371	0.02

*Significant at p>0.05 ; **Significant at p>0.01

Table 4. Meristic count characters of *Coptodon guineensis* expressed as percentage of standard length from the Buguma Creek and the New Calabar River.

Meristic counts	New Calabar River	Buguma Creek	F	t	*p*-value
No of scale along the lateral line	174.38±4.62	202.16±5.08**	0.001	-4.047	0.00
Dorsal fin ray	96.52±2.83	116.98±2.55**	0.576	-5.372	0.00
Dorsal fin spine	124.44±3.31	144.81±3.15**	0.599	-4.457	0.00
Anal fin ray	78.76±2.10	84.39±1.80*	3.008	-2.036	0.04
Anal fin spine	23.99±0.64	27.79±0.60**	0.322	-4.338	0.00
Pelvic fin ray	39.99±1.06	46.31±1.00**	0.321	-4.338	0.00
Pelvic fin spine	8.00±0.21	9.26±0.20**	0.32	-4.339	0.00

*Significant at p>0.05; **Significant at p>0.01

Table 5. R^2 values and beta (B) values for the morphometrics measured against the standard length.

Morphometric Traits	New Calabar River		Buguma Creek		Overall	
	R^2	B	R^2	B	R^2	B
Pre orbital length	0.05	3.21	0.11	2.78	0.08	3.38
Post orbital length	0	-0.68	0.02	0.91	0.00	0.39
Head length	0.02	1.27	0.11	2.03	0.05	1.83
Eye diameter	0.17	1.01	0.01	0.86	0.05	3.78
Pre dorsal fin length	0.02	1.27	0.09	1.91	0.04	1.69
Pre anal fin length	0.88	0.91	0.9	0.98	0.90	0.94
Pre pelvic fin length	0.01	1.09	0.02	0.81	0.03	1.30
Caudal fin length	0.07	2.47	0	0.15	0.11	1.05
Peduncle length	0.48	3.30	0.49	3.22	0.35	3.93
Peduncle depth	0.56	3.52	0.64	4.32	0.62	3.83
Body depth	0.24	0.49	0.37	0.84	0.33	0.59

Table 6. R^2 values and beta (B) values for the meristic counts measured against the standard length.

Meristic counts	New Calabar River		Buguma Creek		Overall	
	R^2	B	R^2	B	R^2	B
Dorsal fin ray	0.002	-0.11	0	0.21	0.01	-0.29
Dorsal fin spine	0.003	0.36	0.01	0.45	0.00	0.27
Anal fin ray	0.017	-1.34	0.01	0.8	0.04	1.38
No of scale along the lateral line	0.002	0.15	0.01	0.07	0.00	0.07

Of the eleven morphometric characters, only two differ significantly (p<0.05) between the two populations, these characters are the head length and Pre-Orbital Length. The remaining nine characters exhibited no significant difference (p>0.05) between the two populations (Table 7).

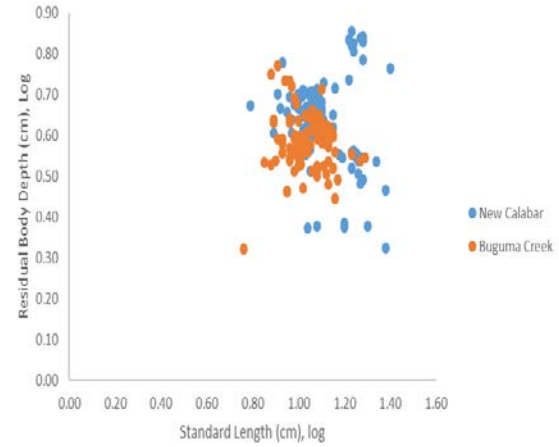
Table 7. R^2 values and F values for the morphometrics measured against the standard length. P<0.05 showed significant variation in the morphometrics of the population.

Morphometric Traits	Df	df_{error}	F	p-value	R^2
Head Length	1	196	4.537	0.034*	0.063
Eye Diameter	1	196	1.549	0.215	0.056
Caudal fin length	1	196	0.079	0.779	0.574
Peduncle length	1	196	2.585	0.109	0.459
Body depth	1	196	1.215	0.272	0.411
Post Orbital length	1	196	8.214	0.005**	0.135
Post orbital length	1	196	3.07	0.081	0.018
Pre dorsal fin length	1	196	3.191	0.076	0.05
Pre anal fin length	1	196	0.449	0.503	0.857
Pre pelvic fin length	1	196	0.494	0.483	0.041
Peduncle depth	1	196	0.001	0.976	0.637

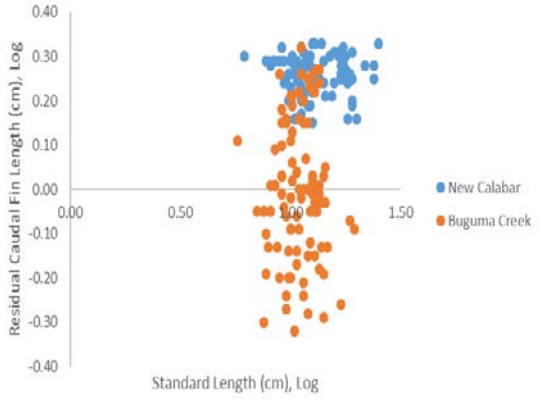
*Significant at p<0.05, **Significant at p<0.01

Analysis of the covariance (GLM on Log transformed data, with SL as covariate) indicated that *C. guineensis* in the two locations differed significantly in the relative body depths (F 1,197df= 16.494; p<0.001), caudal fin length (F 1,197df= 223.92; p<0.001) and peduncle length (F 1,197df= 36.88; p<0.001). But no statistical difference was observed regarding the remaining parameters.

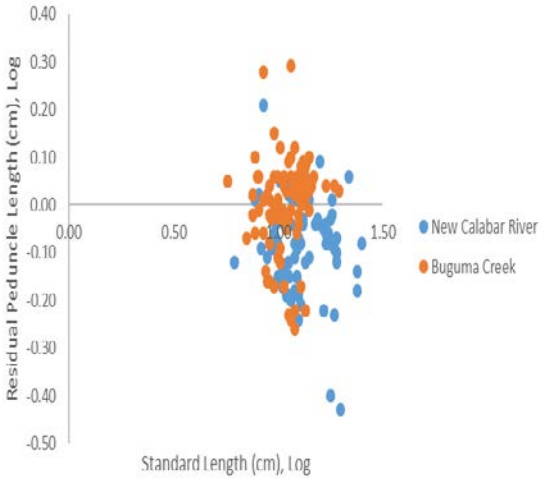
The residual of body depth as well as the residual of caudal fin length plotted against standard length showed a clear differentiation between the species as well as among the stocks. The body depth of *C. guineensis* in the New Calabar River was higher compared to that of the population from the Buguma Creek (Figure 2). On the contrary, the residual of the peduncle length plotted against the standard length showed that the peduncle of the population from the Buguma Creek was longer than that of the New Calabar River population.



a



b



c

Figure 2. Results of residual body depth (a), caudal fin length (b) and peduncle length (c) plotted against standard length in the two sites

4. Discussion

In this study, both morphometrics clearly demonstrated variations among the two populations of *C. guineensis* from the New Calabar River and the Buguma Creek. For example, the total length and total weight of *C. guineensis* from the New Calabar River were higher than those of the Buguma Creek. This may be attributed to environmentally–induced morphological differences. Similar results were recorded regarding all the morphometric measurements apart from the eye diameter and penduncle length that were slightly higher in the population of *C. guineensis* from the Buguma Creek than the New Calabar River. This could be attributed to turbidity and feeding habits of the fish in the Buguma Creek. The eye diameter which contributed heavily to the regional differentiation, may reflect differences in turbidity (Matthews, 1988). Diet has been shown to cause variation in the morphology not only in fish, but also in most organisms (Fermon and Cibert 1998). Water temperature, light cycle and food conditions seem to be the principal environmental factors influencing the growth and reproductive cycle of crustaceans (Pezzack and Corey 1979; Winkler and Greve 2002). Solomon *et al*., (2015) reported that the analyses of morphometric characters revealed abundant variations among different populations. Out of the seven meristic counts examined, only two exhibited slight variations between the two populations and significant difference in dorsal fin ray and anal fin ray between the two populations. Eyo and Mgbenka (1992) found that specific differences in the meristic counts were exhibited in both the anal fin ray and the vertebral count in the Clariids of Anambra River, Nigeria. The divergences in the morphological structures among the populations of fish species are a common biological phenomenon. Swain and Foote (1999) stated that the phenotypic variation in the morphological characters or meristic counts may not only be genetic but may also be environmentally-induced. Krabbenhoft *et al*. (2009) described the environmental factors underlying the morphological changes as water clarity, water depth and flow, food availability and physical complexity. Layman *et al.* (2005) also proposed that the fish assemblage composition in combination with the commercial netting play an important role in the morphological differences.

The meristic counts revealed clearer discriminating differences between the specimens of the two populations than the morphometric measurements. The differences in the morphological and meristic characters of the specimens are supposed to be in association with the aquatic ecosystems from which they originated (Cakić *et al*., 2002; Franičević *et al*., 2005). In this study, the mean head length expressed as the percentage of standard length was slighly higher in the population from the Buguma Creek than that of the New Calabar River. This result came in agreement with Stiassny *et.al.* (2008).

C guineensis from the New Calabar River indicated that the allometric growth was relative to standard length in most of the characters considered apart from PrOL, PL, and PD with the isometric growth pattern. *C guineensis* from the Buguma Creek studied with respect to SL showed that all the external parameters displayed allometric growth expect for PoORL and BD. This implies the changes in the different dimensions of the body parts that are correlated with changes in the whole body. The allometric growth suggests that the weight of the fish increased faster or decreased in relation to the cube of their standard lengths; therefore, adults may appear different

from the young ones (Bagenal and Tesch 1978). All the four meristic characters analyzed did not express any relativeness to the standard length of all the meristic variables studied apart from the influence of size. It has been established that the meristic characters are independent of the fish size; hence, they should not change during growth (Strauss 1985).

5. Conclusion

The morphological variations examined in this paper are preliminary and provide an insight into populations of the same species from two different habitats.There was a clear morphological differentiation between these two groups of fish. The specimens from the Buguma Creek were generally smaller than those from the New Calabar River. Also, both the univariate and the multivariate analyses clearly demonstrate morphological variations among the two populations. The genetic analysis must be conducted on the same species and locations for future researches.

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Rhynchostylis cymifera (Orchidaceae), a New Species from South Western Ghats, India

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Abstract

Rhynchostylis cymifera Yohannan, J.Mathew and Szlach., a new orchid species from Nilambur Forests, part of the southern Western Ghats, India, is here described and illustrated. Taxonomic descriptions, morphological differences to their allied taxa and colour photographs are provided to facilitate identification in the field.

Keywords: New species, Orchidaceae, *Rhynchostylis cymifera*, Western Ghats

1. Introduction

Rhynchostylis Bl. (Orchidaceae, Vandeae) is a genus of epiphytic orchids which comprises only four species distributed in the Indian subcontinent, China, Indochina, Malaysia, Indonesia and the Philippines (Seidenfaden, 1988; Higgins, 2013). All species of the genus are large or medium-sized monopodial plants characterized by thick, stiff, leathery leaves, stout, thick roots and many-flowered inflorescence, either pendulous or erect. The attractive flowers emit a pleasant sweet scent. The simple lip is stiffly connate with the prominent column foot. Basally the lip is elongate into rather spacious, erect spur, more or less laterally compressed. Sepals differ in form and are broadly opened. The gynostemium is rather stout, erect, with elongate rostellum, apical anther is incumbent, operculate with beak-like apex. Two pollinia are subglobose, more or less cleft and are attached to linear, lamellate tegula. The single viscidium is elongate, lamellate, and somewhat sticky on the ventral surface. The species of the genus are rather easily separated from other Vandaceous genera.

Recent floristic exploration in the evergreen forests of Nilambur has yielded several interesting additional specimens of *Rhynchostylis*. The evaluation of these specimens in the literature and in herbaria revealed that some accessions do not belong to any as yet described species (Sasidharan, 2013; Nayar *et al*., 2014). This has resulted in the recognition of a distinct novel species, which is described here as *Rhynchostylis cymifera.*

2. Methodology

An unusual specimen of *Rhynchostylis was* collected by RY and JM in May of 2012 during a botanical survey of Nilambur forests (Malappuram District, Kerala) of South Western Ghats. Here, the climate is moderately cold and elevation is 1200 m from sea level. The specimen considered here was morphologically compared to all known *Rhynchostylis* species with available herbarium materials in K (Royal Botanical Garden, Kew), CAL (Central National Herbarium, Howrah, India), KFRI (Kerala Forest Research Institute Herbarium, India), TBGT (Jawaharlal Nehru Tropical Botanical Garden Herbarium, Keala, India), JCB (Indian Institute of Sciences Herbarium, Bangalore, India) and an in-depth literature survey conducted during the period 2012-2016. Photographs of the new species are provided for better understanding of the morphological diversity. Its material stored in the MSSRF (M.S. Swaminathan Research Foundation Herbarium, Kerala, India)

3. Taxonomic Treatment

Rhynchostylis cymifera Yohannan, J.Mathew and Szlach., *sp. nov.*(Figure1and 2)

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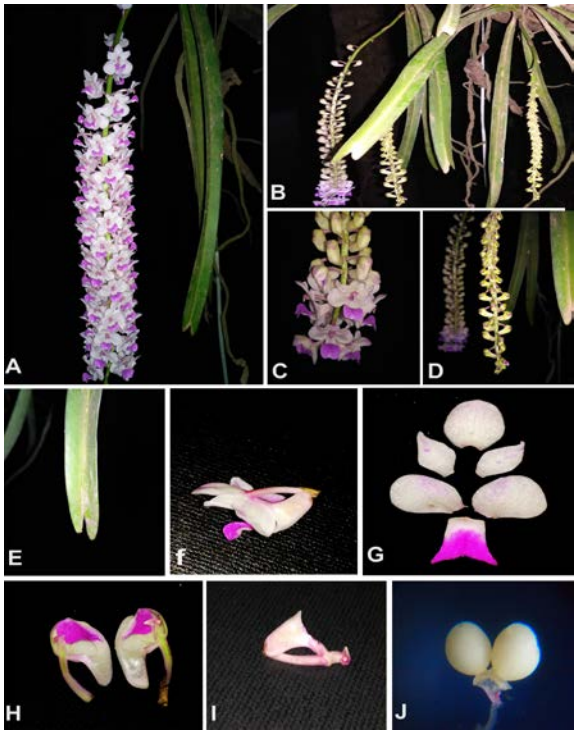

Figure1. *Rhynchostylis cymifera*: **A.** Inflorescence, **B.** Habit, **C & D.** Cymose inflorescence, **E**. Leaf tip, **F.** Flower, **G.** Sepals, petals and lip, **H.** LS of the unopened flower, **I.** lip's spur, **J**. Pollinia.

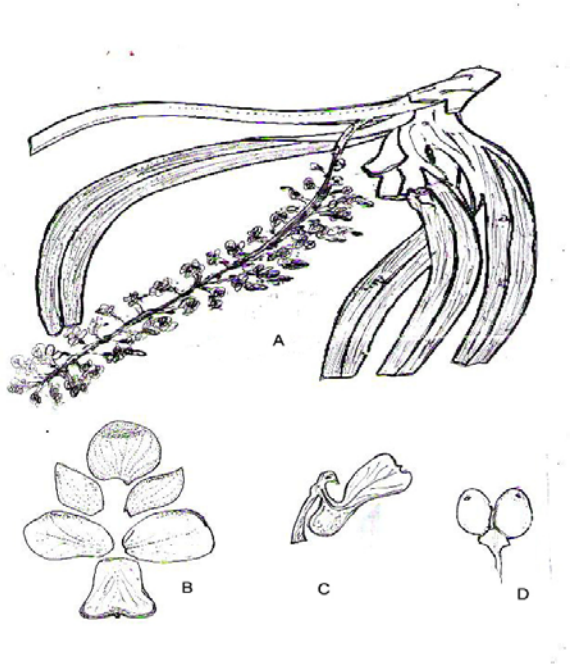

Figure 2. *Rhynchostylis cymifera*: **A. A floweing twig**, **B.** Sepals, petals and lip, **C.** Column with spur, **D**. Pollinia.

3.1. Diagnosis:

Similar to *Rhynchostylis retusa* (L.) Bl. but differing by its odourless cymose inflorescence, rectangular-obovate lip with emarginated apex, obliquely rhombic petals, long pointed rostellum and poratepollinia. Prominent morphological differences distinguishing *Rhynchostylis cymifera* from the allied species are indicated in taxonomic discussion.

TYPE: INDIA: KERALA: Malappuram District, Nilambur, Vaniyampuzha Estate, altitude 1200 m a. s. l., 20 May 2012, *Yohannan 4417* (holotype: TBGT, isotype: MSSRF!, UGDA-DLSz! - photos)- since the herbarium of the M.S. Swaminathan Research Foundation (Kalpatta, Wayanad, Kerala, India) has not been formally registered with Index Herbariorum(Thiers, 2017), it is here referred to as "MSSRF".

3.2. Etymology:

The specific epithet (viz. *cymifera*) refers to the cymose inflorescence, a clear peculiarity of this species.

Herbs epiphytic, monopodial. Stems short, stout, ascending, enclosed in leaf sheaths with thick roots ca30-40 cm long. Leaves 7-10, 20-25 x 1.6-2 cm, alternate, long and narrow, thickly fleshy, channeled, sheathing and articulate at base, very unequally bilobed or toothed. Inflorescences1-3, 30-34 cm long, lateral, pendulous, cymose, basipetal, densely many flowered; peduncle and rachis stout and thick, peduncle 7-9 cm long, bracts 3-4, obovate to lanceolate, Floral bracts lanceolate, not reflexed, membranous, persistent. Pedicel with ovary7-10 mm long. Flowers non-resupinate, medium-sized, broadly opened, odorless, white with pink suffusion, lip purple in apical half. Dorsal sepal 7-9 x 5-6 mm, elliptic-ovate, rounded at apex. Lateral sepals 8-10 x7-8 mm, oblong obovate, rounded at the apex, oblique at base. Petals 4-6 x 3-4 mm, obliquely rhombic, acute at the apex. Lip 9-13 x 6-9 mm, stiffly connate with the column foot, rectangular-obovate in general outline, apex emarginate, spurred at base; spur 5-6 x 3-4 mm, broadly cylindrical, laterally compressed, obtuse and backward-pointing. Gynostemium ca 3-4 mm long, short, with a prominent foot; anther cap long pointed; rostellum long pointed; pollinia2, globose, porate; stipe long and narrow, apex slightly dilated; viscidium oblong ovate. Stigma deep-seated. Fruit not seen.

3.3. Phenology:

Flowering recorded in May-June

3.4. Additional Specimen Examined:

INDIA: KERALA: Malappuram District, Nilambur, Punchakolli Estate, altitude 1300 m a. s. l., 22 May 2016, *Yohannan 4419, 4420 & 4421*(MSSRF!).

3.5. Distribution and Ecology:

Rhynchostylis cymifera grows in the deciduous forests (altitude ± 1200 m) of the Nilambur Forests, Malappuram, Western Ghats, Kerala, India. It grows on the trunks of *Xylia xylocarpa* (Roxb.) Taub.in association with some bryophytes. This host species is an Asiatic element, occupying a very restricted area in the forests of Nilambur.

3.6. Conservation Status:

Field surveys have located about nine individuals within the Punchakolli and Vaniyampuzha Estates Hills covering an area of 50 km^2. The threat status of this species has been assigned as 'Critically Endangered' as per the guidelines of IUCN (2001). Based on the above observations, adequate measures should be adopted to ensure the protection of this species in its natural habitat. Moreover, further surveys of this species are required. Apart from habitat destruction caused by anthropogenic

intervention and wild fires during the summer, no other specific threats were determined during the field studies.

4. Taxonomic Discussion

Rhynchostylis cymifera can be easily distinguished from all other species of the genus by cymose, basipetal inflorescence, the character rarely found in orchids. In habit, it is similar to both *R. retusa* (L.) Bl. and *R. coelestis* Rchb.f. and share with them narrow, linear and long leaves. It can be easily separated from Indochinese *R. coelestis* by numerous morphological characters from which pendulous inflorescence (vs erect inflorescence) is the most eye-catching. It appears that *R. cymifera* can be related to *R. retusa*. Despite the latter, however, the lip of our new species is emarginated (vs shortly acute), petals are obliquely rhombic with acute apex (vs oblong, rounded at apex), dorsal sepal is rounded (vs acute), tepals are evenly-white with pink suffusion (vs white or pink but with few purple spots) and pollinia are porate (vs cleft). According to our records, the spur of *R. cymifera* is broadly cylindrical and in *R. retusa* – conical – cylindrical with much higher orifice. Moreover, the lip lamina of the new entity is longer than spur, whereas in *R. retusa* lip lamina is shorter than spur. It is noteworthy that *R. cymifera* is the only species of the genus with odorless flowers. In our opinion all these characters give good premises for describing *R. cymifera* as separate species.

4.1. Key to the genus Rhynchostylis

1. Inflorescence upright ascending, tip of spur distinctly bent downwards.....*R. coelestis*
1: Inflorescence drooping, spur not bent 2
2. Lip apex distinctly 3- lobed, column foot absent.....*R. gigantea*
2: Lip apex not distinctly 3- lobed, column foot present.....3
3. Lip's apex upwards and hook like, spur's tip truncate.....*R. rieferii*
3: Lip's apex downwards, spur's tip rounded.....4
4. Racemoseinflorescence, lip lamina is shorter than spur..... *R. retusa*
4: Cymoseinflorescence, lip lamina is higher than spur..... *R. cymifera*

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Molecular Detection of Putative Virulence Factors (Fungalysin and Subtilisin) in *Aspergillus tamarii* Isolated from Human Skin

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Abstracts

The genus *Aspergillus* is widely distributed containing a number of species that are opportunistic pathogens for both humans and animals. Some of the species of this genus were identified based on morphology. However, the molecular detection of the pathogens is necessary. The detection of the virulence factors of *Aspergillus tamarii* associated with the human skin was performed in this study. *Aspergillus tamarii* isolated from skin infections were examined using morphological and molecular criteria. The phylogenetic relationship of isolates was determined using sequences from the ITS gene. Fungalysin (*MEP3*) and subtilisin (*SUB1*) genes were also detected in the isolates using Polymerase Chain Reaction. Out of ten morphologically identified *Aspergillus tamarii* seven were identified to be *Aspergillus tamarii* using molecular methods. All the molecularly identified *A. tamarii* had fungalysin (*MEP3*) gene while the subtilisin (*SUB1*) gene was detected in six out of seven isolates. The nucleic acid sequencing offers an additional tool for a better diagnoses and understanding of the phylogenetic relationships within genus or species of pathogenic microbes.

Keywords: Aspergillosis, *Aspergillus tamari*, Cutaneous infections, Fungalysin, Subtilisin

1. Introduction

Not until the recent time, aspergilloses have not been reported to be rare opportunistic fungal infections. The *Aspergillus* species have been implicated in both localized and invasive infections of man with a higher occurrence especially among immunocompromised patients (Mustafa *et al.,* 1992; Walmsley *et al.,* 1993; Klein and Tebbets, 2007). Cutaneous aspergillosis has been reported to be more frequent among burns victims, neonates and children. Individuals with cancer, bone marrow and solid organ transplant recipients have also been reported to be disposed to cutaneous aspergillosis (Martin *et al.,* 2003). Surgical wounds (Brock, 2009), traumatic inoculation (Gupta *et al.,* 2001; Latge and Calderone, 2002), or exposure to high spore counts in occupations such as farming (Chakrabarti, 2005) have been identified as pre-disposing factors for healthy individuals to contract cutaneous aspergillosis. Cutaneous aspergillosis may produce pustules or lesions with purulent discharge which generally are common among neonates (Granstein *et al.,* 2005). *Aspergillus fumigatus, A. flavus,* and *A. niger* are the prominent members of the genus that have been implicated in most cases of aspergillosis (John and Shadomy, 1987; Grossman *et al.,* 2005). Non-*flavus* and –*fumigatus Aspergillus* rarely cause cutaneous lesion.

Some dermatophytes have been reported to produce fungalysin (an endometalloprotease) which has been recognised as keratin-induced proteases that expresses during fungus-keratinocyte interaction (Brouta *et al.,* 2001; Tarabees *et al.,* 2015). Fungalysin has been reported to have pronounced collagenolytic, elastinolytic and keratinolytic activities that contribute to the virulence of the secreting fungi (Jousson *et al.,* 2004). *Aspergillus* spp. produce and secrete various hydrolytic enzymes including subtilisin. Subtilisin is an extracellular-located enzyme that is also connected with the cell wall. It generally contributes to the fungal virulence and facilitates tissue colonization. There is a considerable correlation between their activity and the severity of infections (Alp and Arikan, 2008). Subtilisin has the ability to degrade protein (Hogan *et al*., 1996), and specifically attacks elastin, collagen, fibrin and fibrinogen in the tissue of the host (Moutaouakil *et al*., 1993; Tomee and Kauffman, 2000).

There is dearth of information on the association of *A. tamarii* with skin infections and the virulence factors of the fungus isolated samples from the study area. Therefore, this study was conducted to investigate the association of *A. tamarii* with human skin infections, and to detect the

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presence of fungalysin and subtilisin, two putative virulence factors in the organism.

2. Material and Methods

2.1. Sample Collection, Macroscopic Examination and Cultural Identification of the Isolates

Scrapings or swabs were collected from children [(n=23) aged 5-14 years] with different skin infections, and were plated on Saboraud Dextrose Agar supplemented with chloramphenicol. They were incubated for five days at 25 ^{o}C. The fungal identification was carried out based on microscopic examination (growth characteristics, pigment formation). A wet preparation of each colony was prepared using lactophenol cotton blue solution on a clean grease-free slide as described by Raper and Fennell (1965). Fungal spore and hypha were picked on the slide, lactophenol blue was added, and a cover slip was used. The samples were examined under the microscope by X10 and X40 objective lenses.

2.2. Molecular Identification

The identities of the isolates were confirmed further using polymerase chain reaction. The DNA extraction was carried out on the isolates using the Zymo Fungal/Bacteria DNA extraction kit according to the manufacturer's instructions. The purity and concentration of the extracted DNA was evaluated using a nanodrop (ND 1000) Spectrophotometer (Thermo Scientific, USA). All the samples showed a DNA yield between 5ng - 25ng, and the extracted DNA was optimally pure showing A_{260}/A_{280} between 1.60-1.80.

Polymerase chain reaction was carried out to amplify the ITS gene of the fungus using the primer pair ITS-1 (5'-TCCGTAGGTGAACCTGCGG) and ITS-4 (5'-TCCTCCGCTTATTGATATGC). The PCR reaction was carried out as described earlier.

Eppendorf Vapo protect thermal cycler (Nexus Series) with an initial denaturation of 95 °C for fifteen minutes followed by 35 amplification cycles of thirty seconds at 95 °C; one minute at 58 °C and 1.5 minutes at 72 °C was used for the thermal cycling. The final extension step of ten minutes at 72 °C was followed. The amplification product was separated on a 1.5 % agarose gel, and electrophoresis was carried out at 80 V for one hour and thirty minutes. The DNA bands were visualized by ethidium bromide staining. One hundred base pair DNA ladder was used as DNA molecular weight standard.

Purified PCR products were sequenced with Exo sap, and were sent to Epoch Life science (USA) for Sanger sequencing. The consensus sequences obtained from both primers were edited and subjected to BLAST searches to assign tentative identity and phylogenetic inference.

2.3. Determination of the Presence of Pathogenic Factors Using PCR

DNA extraction was carried out from the isolates using the Zymo Fungal/Bacteria DNA extraction kit according to the manufacturer's instructions. The purity and concentration of the extracted DNA was evaluated using a NANODROP (ND 1000) Spectrophotometer (Thermo Scientific, USA). All the samples showed a DNA yield between 5 ng – 25 ng, and the extracted DNA was optimally pure showing A_{260}/A_{280} between 1.60-1.80.

2.4. PCR Amplification of Fungalysin (MEP3) and Subtilisin (SUB1) Genes

Polymerase chain reaction was carried out to amplify the *MEP3* gene and *SUB1*of the fungi using the primer pair *MEP*3 forward 5' GCCATGTCCTTCTCCAAG 3' reverse 5' AGACCACGCTTAGCAAAG 3' and *SUB1* forward 5' ATCCTGTCTATGCCTCATG 3', reverse 5' AATCGAAGTCGAAGTTATC 3' as reported by Lemsaddek *et al.* (2010) and Alp and Arikan (2008) respectively. The PCR reaction was carried out using the Solis Biodyne 5X HOT FIREPol Blend Master mix. PCR was performed in 25 µl reaction containing 1X Blend Master mix bufferBuffer (Solis Biodyne), 1.5mM $MgCl_2$, 200 µM of each deoxynucleoside triphosphates (dNTP)(Solis Biodyne), 25pMol of each primer (BIOMERS, Germany), 2 unit of Hot FIREPol*DNA* polymerase (Solis Biodyne); However, additional Taq DNA polymerase was incorporated into the reaction mixture to reach 2.5 final units. Proof reading enzyme, 2 ng of the extracted DNA, and sterile distilled water was used to complete the reaction mixture.

Thermal cycling was conducted in an Eppendorf Vapo protect thermal cycler (Nexus Series) as described earlier. The amplification product was separated on a 1.5 % agarose gel, and electrophoresis was carried out at 80 V for one hour. After electrophoresis, DNA bands were visualized by ethidium bromide staining. 100 bp DNA ladder was used as DNA molecular weight standard.

3. Results

After culturing on Glucose Peptone Agar (supplemented with chloramphenicol to inhibit the growth of any bacteria from the culture), only ten out of the twenty-three samples were positive for *Aspergillus* species. The Isolates, that produce abundant conidia heads in dull yellowish green shades, becoming metallic bronze at maturity with diameters between 6.0 and 7.0 cm in eight days when cultured at 37 ^{o}C, were suspected to be *A. tamarii.*

The genomic DNA extracted from all the ten fungi isolates was identified by PCR analysis. Seven were identified to be *A. tamarii* and others were one *A. nomius,* one *A. aculeatus* and one *Pichia kudriavzevii.* Only seven that were positive for *A. tamarii* were chosen for this study.

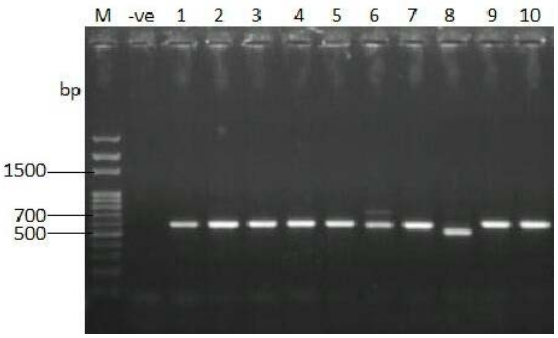

Figure 1. Gel electrophoresis of PCR amplification of *ITS* gene, Lane M is a 1500 bp ladder, Lane -ve is the negative control lane while Lanes 1 to 10 are morphologically identified *Aspergillus tamarii* isolated from human skin

Table 1. Sequence spacing significant alignment of the isolates isolated from skin infection

Isolates	Max score	Total score	Query cover (%)	Identity (%)	Accession number	Identification
A	11024	1145	96	99	KC874831.1	*Aspergillus tamarii*
B	1011	1011	75	99	JQ257030.1	*Aspergillus tamarii*
C	1061	1061	63	96	KR905619.1	*Aspergillus nomius*
D	1022	1363	85	99	NR135325.1	*Aspergillus tamarii*
E	1016	1400	87	99	MR135325.1	*Aspergillus tamarii*
F	1016	1158	95	99	JQ257030.1	*Aspergillus tamarii*
G	952	1376	61	97	KJ706301.1	*Pichia kudriavzevii*
H	1271	2086	94	95	KP965728.1	*Aspergillus aculeatus*
I	1014	1193	97	99	KP784375.1	*Aspergillus tamarii*
J	1016	1016	54	99	JQ257030.1	*Aspergillus tamarii*

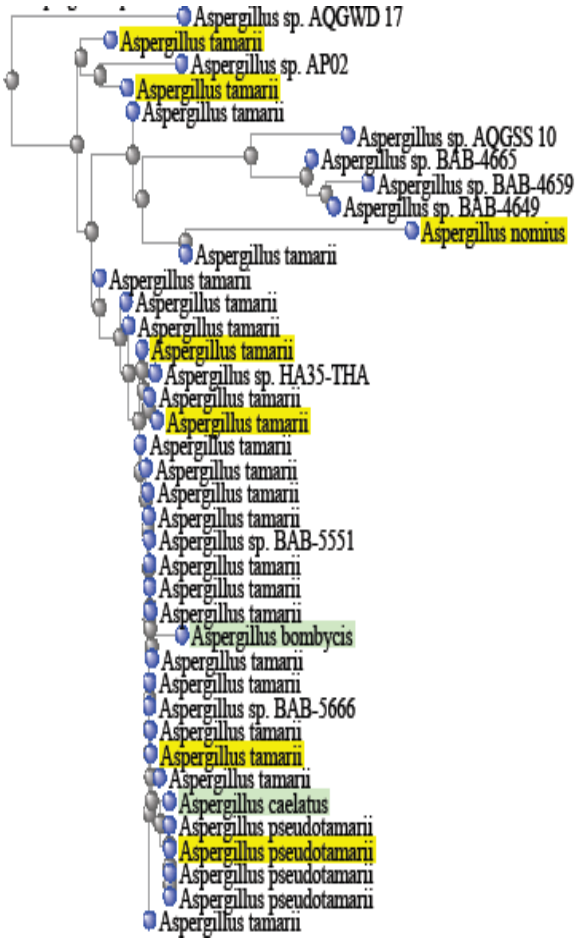

Figure 2. Phylogenetic tree of *Aspergillus tamarii* constructed from aligned combined DNA sequences of the ITS gene. Sequences obtained during this study are presented in yellow.

3.1. Determination of Some Pathogenic Factors from the Isolates

The seven selected *Aspergillus tamarii* isolates were tested for the presence of two virulence factors, and they are *MEP3* and *SUB1* gene. For the polymerase chain reaction, all the seven isolates showed amplification using keratin, elastin and collagen (*MEP3*) gene with amplification band of 850 bp and fungilisin and subtilisin (*SUB1*) gene primers with amplification band 1250 bp (Figure 2 and 3).

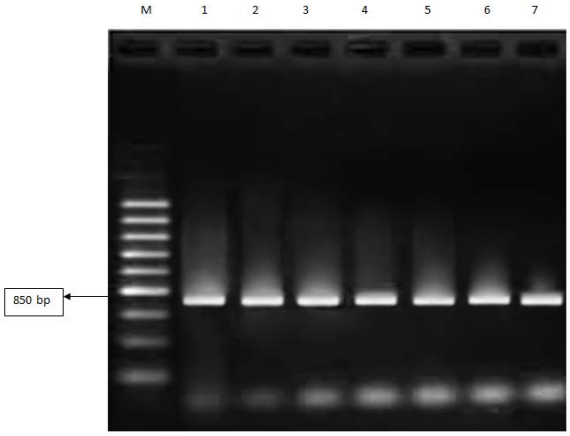

Figure 3. Gel electrophoresis of PCR amplification of *MEP3* gene, Lane M is a 1500bp ladder. Lanes 1 to 7 are *Aspergillus tamarii* isolated from human skin

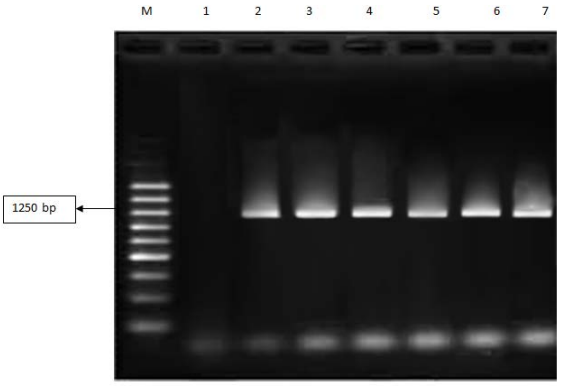

Figure 4. Gel electrophoresis of PCR amplification of *SUB1* gene. Lane M is a 1500 bp ladder. Lanes 1 to 7 are *Aspergillus tamarii* isolated from human skin

4. Discussion

Out of the twenty three samples collected only ten were identified as *Aspergillus tamarii* based on their growth characteristics, pigment formation, and gross colony morphology identification features. Further molecular identification, PCR showed that only seven out of the isolates were *Aspergillus tamarii*, one of each was *Aspergillus nomius*, *Aspergillus aculeatus* and *Pichia kudriavzeuii*. This further proved the specificity and reliability of molecular identification over the conventional methods of fungi identification (Tomee and Kauffman, 2000). The infection caused by the organisms maybe as a result of low immunity of the children or malnutrition. Constant contact with the soil, the reservoir of *Aspergillus* spp. may also increase the chances for the infection by the

organisms among children (Casadevall, 2007; Ganaie *et al.*, 2010).

The presence of the fungalysin (*MEP3* gene) and subtilisin (*SUB1* gene) in *Aspergillus tamarii* is an indication of the degree of pathogenicity of the organism as reported by Tomee and Kauffman (2000). All the isolated *Aspergillus tamarii* harbored *MEP3* gene, and six out of the seven strains screened has the *SUB1* gene. These putative factors have already been characterized and shown to be responsible for the elastinolytic, keratinolytic and collaginolytic activities of the fungus (Tomee and Kauffman, 2000; Brouta *et al.*, 2001; Baldo *et al.*, 2010). Keratin protects the epithelial cells from damage or stress (Gupta and Ramnani, 2006), while collagen assists the connective or fibrous tissues (Chen *et al.*, 2009). The elastase production by the organism assists it to degrade the connective tissue that assists the tissues in the body to snap back to their original shape after being stretched or contracted (Almine *et al.*, 2010).

The *MEP3* genes codes for keratinolytic activity which will allow the fungus to degrade one of the most important constituents of the skin, nails and hair. *Aspergillus tamarii* have been reported to infect the eyelids and are responsible for onychomycosis (Lise *et al.*, 2004). *Aspergillus tamarii* have been isolated from the skin for the first time in Ekiti State of Nigeria. The presence of *MEP* and *SUB* genes in *A. tamarii* indicated that it is a dermatophytes to watch out for in the clinical practice.

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In Vitro Multiplication of the White Wormwood, *Artemisia herba-alba asso*

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Abstract

Artemisia herba-alba is a wild aromatic medicinal shrub which grows in the arid areas of North Africa and the Middle East. It is heavily subjected to loss and threats due to heavy grazing, cultivation and collection by the people who used it in folk medicine. *In vitro* propagation was experimented with different combinations of plant growth regulators to develop a suitable protocol to propagate this plant. *In vitro* microshoots of *A. herba-alba* were initiated from seeds. A maximum germination of seeds (94 %) was obtained using Paper-Bridge inserted in half Murashige and Skoog (MS) media and supplemented with 15 g/L of sucrose and 1.0 mg/L Gibberellic Acid (GA_3). Proliferation of the *in vitro* plant was experimented at different concentrations (0.0, 0.5, 1.0, 1.5 and 2.0 mg/L) of 6-Benzylaminopurine (BAP), kinetin, or 6-(gamma, gamma- Dimethylallylamino) purine (2iP). Rooting was experimented at different concentrations (0.0, 0.5, 1.0, 1.5 and 2.0 mg/L) of, Indole-3-acetic acid (IAA), Indole-3-butyric acid (IBA) or alpha-Naphthalene acetic acid (NAA). The maximum proliferation of the *A. herba-alba* (23.6) microshoots was obtained when MS medium was supplemented with 1.0 mg/l 2iP and 1.5 mg/L GA_3. The maximum root number (18.8) and length (14.08 cm) was achieved using 0.5 mg/L IBA. Both IAA and NAA failed to promote root induction. The rooted plants acclimatized successfully with 50 % of survival and were grown in the greenhouse. These results indicate the enormous potential of *A. herba-alba* for a large-scale multiplication, and it represents the first step to conserve its germplasm.

Keywords: *Artemisia herba-alba*, White wormwood, Shih, *In vitro* culture, Micropropagation, Seeds germination

1. Introduction

Artemisia herba-alba belongs to the Asteraceae family. It is a perennial dwarf shrub that grows in arid and semi-arid climates. It is an attribute of the steppes and deserts of the Middle East, North Africa, Spain, extending into the north western Himalayas (Haouari and Ferchichi, 2009; Hedi *et al*., 2010). *A. herba-alba* is a native plant of Jordan (locally known as Shih). This plant is used locally in folk medicine due to its high content of essential oils, sesquiterpene lactones, and other chemical compounds (Aburjai *et al*., 2007; Mohamed *et al.*, 2010; Sharaf *et al*, 2012).

Due to the increased demand and consumption of *A. herba-alba* for medicinal purposes, the spreading out of urbanization and overgrazing, there is a need to conserve this valuable genetic resource from extinction through mass production. Plant tissue culture techniques are considered the most effective methods for propagating a high number of plant species (George, *et al*., 2008). Stem cuttings and seed multiplication methods are not always suitable to all conditions depending highly on the species (Gurib-Fakim, 2014). Plant tissue culture enables the mass propagation of uniform plants, and helps overcome the problems of propagation. Hundreds or even thousands of rooted plants will be successfully acclimatized from a few plant materials in a short time. Different levels of various plant growth regulators (PGRs) were used in the previous studies to induce proliferation and rooting *in vitro* for some medicinal plants (Al- Qudah, *et al*., 2011; Evenor and Reuveni, 2004; Mostafa, *et al*., 2010; Musallam, *et al*., 2011; Owies, *et al*., 2009). The application of the micropropagation technique is to multiply plants rapidly in a short time. This technique produces plants that are more likely to maintain genotypic and phenotypic fidelity to the original clone (George, *et al*., 2008; Hofman, *et al*., 2002; Machakova, *et al*., 2008; Shatnawi, *et al*., 2007; Staden, *et al*., 2008).

Different *Artemisia* species were *in vitro* propagated using micropropagation or organogenesis, such as *A. scorpia* (Aslam *et al*., 2006); *A. vulgaris* (Govindaraj *et al.*, 2008); *A. mutellina* (Mazzetti and Donato, 1998); *A. annua* (AL Maarri and Xie, 2010). According to the Jordan Plant Red List (Taifour and El-Oqlah, 2014), *A. herba-alba* was among the LC (least concern) endangered

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species. However, without the efforts and the interests in its multiplication, *A. herba-alba* may enter the endangered species list.

This study is aimed at utilizing tissue culture techniques for the sake of rapid multiplication of the *A. herba-alba* by developing a protocol for *in vitro* multiplication, rooting and *ex vitro* acclimatization of *A. herba-alba.*

2. Materials and Methods

2.1. Seeds Germination

Seeds of *A. herba-alba* were obtained from the National Center for Agricultural Research and Extension (NCARE). The seeds were surface-sterilized by washing thoroughly under running tap water for fifteen minutes with a few drops of mild detergent, antibacterial soap. They were then dipped in an antiseptic solution of 0.6 % sodium hypochlorite for five minutes, followed by washing under running tap water for fifteen minutes. After that the seeds were transferred into ethanol 70 % (v/v) for thirty seconds, and were rinsed with sterile distilled water three times (fifteen minutes each) under laminar airflow cabinet. The following media treatments were used to test the germination percentages:

MT1: Control: Agar media: the seeds of *A. herba-alba were* sowed on water media with 8 g/L agar in an aseptic plastic Petri dish.

MT2: Paper-Bridge on MS liquid media: The seeds were sowed on the top of a filter paper bridge inserted in MS (Murshige and Skoog, 1962) liquid medium for germination.

MT3: Paper-Bridge on half MS liquid medium: The seeds were sowed on the top of a filter paper bridge inserted in half MS liquid medium.

MT4: Paper-Bridge on half liquid MS with Gibberellic Acid (GA_3) media: The seeds were sowed on the top of a filter paper bridge inserted in half MS liquid medium.

MT5: Gibberellic Acid (GA_3) water media: The seeds were sowed on water media supplemented with 1 mg/L GA_3 and solidified with 8 g/L agar dispensed in a small aseptic plastic Petri dish.

MT6: Gibberellic Acid (GA_3) with solid full MS media: The seeds were sowed on MS media with 1 mg/L GA_3 and solidified with 8 g/L agar, dispensed in a small aseptic plastic Petri dish.

MT7: Gibberellic Acid (GA_3) with half MS media: The seeds were sowed on half MS media with 1 mg/L GA_3 and solidified with 8 g/L agar dispensed in a small aseptic plastic Petri dish.

Data on the percentage of seed germination were recorded after six weeks.

For further growth, i*n vitro* seedlings were transferred to the growth room under conditions of sixteen hours of light / eight hours of dark according to the photoperiod regime, photosynthetic photon flux density (PPFD) = 40-45 µ mol. /m^2 /sec), and a temperature of 24 ± 1 °C). Afterwards, the aseptically-grown cultures were directly sub-cultured to MS media without growth regulators for further growth. In order to establish mother stock plants, microshoots (5-7 mm) from seedlings were subcultured on MS medium with 1.0 mg/L Benzylamino purine (BAP), and 1.0 mg/L GA_3. Subculturing was performed by transferring the microshoots (5-7 mm) to a fresh medium every four weeks.

2.2. In vitro Multiplication

Shoot tips (STs) of the microshoot (5-7 mm) were subcultured in Erlenmeyer flasks (250 mL) with 100 mL of solid MS medium containing 3 % (w/v) sucrose, 0.8 % (w/v) agar supplemented with different concentrations (0.0, 0.5, 1.0, 1.5, and 2.0 mg/L), of BAP, Kinetin (6-Furfurylaminopurin) or 6-(Dimethylallylamino)-purine (2iP) with 0.1 mg/L 1-nephthaleneacitic acid (NAA), for a multiple shoot induction. In another experiment the microshoots (5-7 mm) were subcultured on MS medium supplemented with different concentrations of GA_3 (0.0, 0.5, 1.0, 1.5, 2.0 and 3.0 mg/L) to study the effect of gibberellic Acid (GA_3) on the microshoot proliferation. Also, GA_3 at 1.0 mg/L was used with different concentrations (0.0, 0.5, 1.0, 1.5 and 2.0 mg/L) of BAP, 2iP or Zeatin for their effect on multiplication. Data were recorded after six weeks including the number of proliferated microshoots, the microshoot height, callus formation and root formation.

2.3. In vitro Rooting

The microshoots were grown on hormone-free MS medium for one week to eliminate any carry over effect of any hormones that might affect the rooting. To induce rooting, individual microshoots (5-7 mm long) were isolated and transferred to 25×150 mm culture tube containing 12 mL agar-gelled MS rooting medium supplemented with different concentrations (0.5, 1.0, 1.5, and 2.0 mg/L) of IBA(Indol-3-butric acid), NAA or IAA (Indol-3-acetic acid). One set of cultures in which the seeds were sowed on basal MS medium without the addition of auxins and were kept as control. Six weeks later, the cultures were evaluated on the basis of the number and length of the roots as well as the shoots height.

2.4. Ex vitro Acclimatization

The plantlets with well-developed roots were removed from the culture medium. After washing the roots gently under running tap water (to remove the adhering medium), the plantlets were transferred to plastic cups (10-cm diameter) containing a growing medium (1 peat: 1 perlite) mixture. Each cup was covered with a perforated plastic bag to reduce evaporation, and was irrigated with distilled water every two days for three weeks followed by tap water for two weeks. After that, the survival percentage was recorded, and the acclimatized plantlets were maintained for four more weeks in greenhouse conditions at a temperature of 33 ±1°C.

2.5. Experimental Design and Statistical Analysis

The experiments were arranged in complete randomized design (CRD) with ten replicates for each treatment. The data were analyzed using SAS program and analysis of variance (ANOVA). Means were separated using the Least Significance Difference (LSD) at 0.05 probability level.

3. Results and Discussion

3.1. Seed Germination

Germination started after one week in all culture media, and the radical emergence was evaluated as a main indicator for the seed germination percentage. Full MS medium decreased the germination percentage of *A. herba-alba* seeds (Table 1). The high concentration of MS salt affected the germination percentage of *A. herba-alba* negatively; this is may be attributed to the fact that an increase in the osmotic potential prevents the imbibition of water and germination (Kaufman, 1969). The results showed that the highest germination (94 %) was obtained using paper bridge with half MS liquid media containing 1 mg/L GA_3, followed by (90 %) germination on half MS solid media containing 1 mg/L GA_3, (86 %) germination on solid MS media, and (82 %) germination on water and GA_3 media.

In vitro germination of most seeds was achieved by the use of Murashige and Skoog, (1962) medium. In this experiment, the paper bridge with half MS liquid and Gibberellic Acid (GA_3) medium yielded the highest germination percentage (94 %) as shown in Figure 1. Supplementing GA_3 in the germination medium enhanced the germination percentage of *A. herba-alba* seeds in both MS and ½ MS media, indicating that GA_3 promotes the seed germination. Iglesias and Babiano (1997) reported that Gibberellic acid (GA_3) affected the primary dormancy by inducing germination. Similar results were achieved when supplementing the medium with 2.0 mg/L Gibberellic Acid (GA_3) for Persian oregano "*Origanum vulgare* L." and Arabian oregano "*Origanum syriacum* L." (Arafeh *et al.*, 2006).

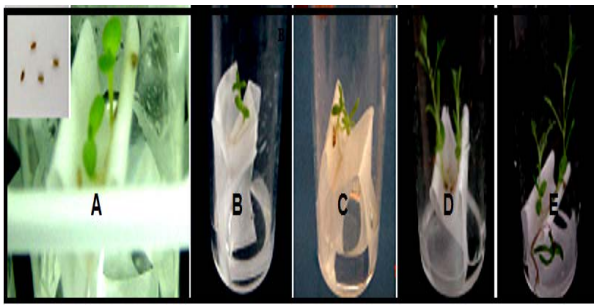

Figure 1. Seedlings of *Artemisia herba-alba* germinated on paper bridge with half MS liquid and Gibberellic Acid (GA_3) medium. (A-E): Seedlings after 1-5 weeks; respectively.

Table 1. Effect of different media on seed germination percentages of *Artemisia herba-alba*.

Germination Medium	Seed germination %
MT1: Control: Water media solid medium	48 c*
MT2: Paper-Bridge + MS liquid medium	28 d
MT3: Paper-Bridge + half MS liquid medium	54 c
MT4: Paper-Bridge + half MS liquid medium	94 a
MT5: Gibberellic Acid (GA_3) water media	82 ab
MT6: Gibberellic Acid (GA_3) MS solid medium	86 ab
MT7: Gibberellic Acid (GA_3) half MS media	90 ab

*Means within column followed by the same letter (s) are not significantly different using LSD at 0.05.

A. herba-alba responded significantly to the increased BAP concentrations up to 1.0 mg/L (Table 2). The shoot length and the number of microshoots, produced from the cultures on MS medium supplemented with (0.0, 0.5, 1.0, 1.5 and 2.0) mg/L BAP, were significantly different. The maximum shoot height (2.5 cm) was obtained from control treatment. Formation of callus occurred at the basis of treatments (including the control), but the largest occurred at 1.5 $mg.L^{-1}$ BAP. The best concentration of BAP which produced the highest shoot number (16.4 microshoots per explants) was 1.0 mg/L BAP. BA has been used to induce multiple shoots in *A. pallens* (Sharief and Chandra, 1991), and *A. vulgaris L.* (Sujatha and Kumari, 2007). Results of Al-Qudah *et al.*, (2011) also showed successful *in vitro* proliferation (8.8 shoot per explants) of *Teucrium polium* L. by using 2.0 mg/L of BAP combined with 0.1 mg/L NAA. Moreover, (Shibli *et al*., 2006) reported that higher BAP concentrations (1.5-2 mg/L) increased the number of microshoots, but it decreased the shoot length of *In vitro* propagated apple-root stock (MM.106). Similarly, *O. vulgare* explants were reported to give the highest shoot number on MS medium supplemented with high levels (1.6 and 2.0 mg/L) of BAP (Arafeh *et al.*, 2006).

Significant variations were obtained among concentrations of kinetin on *in vitro* shoot multiplication of *A. herba-alba* (Table 2). A low concentration of kinetin (0.5 mg/L) gave the highest number of shoots (8.20) of *A. herba-alba*, however, increasing the concentration of kinetin to 1.5 and 2.0 mg/L significantly reduced the number of shoots per explant. The highest shoot length (2.5 cm) was obtained at the control. Adding kinetin to the media negatively affected the shoot length of *A. herba-alba*. In addition to the control treatment, callus formation was only found in the medium supplemented with 0.5 mg /L kinetin indicating that higher kinetin in the culture medium of *A. herba-alba*, suppressed the callus formation. In contrast, Musallam *et al.*, (2011) reported highest callus percentage 90 % in the explants of *Capparis spinosa* on medium supplemented with a high concentration (2.0 mg/L) of kinetin. The highest shoot length (2.9 cm) of *Teucrium polium* L. was also obtained at a low level (0.4 mg/L) of kinetin (Al-Qudah *et al.*, (2011). However, the high concentration of kinetin oppositely affected the proliferation of some plants. For example, Bouhouche and Ksiksi, (2007) reported that, the highest *in vitro* proliferation rate of *Teucrium stocksianum* Boiss was achieved on medium containing 3 mg/l kinetin and 0.5 mg/L IAA. A maximum shoot induction and the number of microshoots /explants were reported for *Pandorea jasminodes* when using 1 mg/L kinetin (Kancherla and Bhalla, 2001). The highest number (3.9) of shoots, shoot length (5.5 cm), and number of leaves/explants (26.2) of *O. vulgare* were obtained when the media were supplemented with 2.0 mg/L of kinetin (Arafeh *et al.*, 2006).

The best multiplication parameters and growth performance of *A. herba-alba* were obtained using 1.0 mg/L 2iP (Table 2, Figure 2), where the maximum number of the microshoots per explants was 22.9, and the longest shoots were obtained (2.61 cm) (Table 2). Callus formation appeared at all 2iP treatments, but increasing 2iP up to 2 mg /L reduced the callus size. This may be attributed to the activity of the plant hormone (2ip,

cytokinin) that exhibits either synergistic or antagonistic interactions at the cellular level (Danova *et al.*, 2017). Accordingly, this study suggests that the genotypic factor seems to be operating in response to particular concentrations of growth regulators (Lomin *et al.*, 2015). Higher concentrations of cytokinins more than 1.0 mg/L reduced the shoot numbers as well as the shoot length. It is well-known that exogenously supplied plant-growth regulators; strongly affect patterns of the plant growth and development in vitro. Logically, plant-growth regulator treatments at low or high concentrations have an impact on endogenous phytohormone homeostasis in the plant through multiple mechanisms (Danova *et al*., 2017). Cytokinin binding at specific concentration effects the plant growth depending on many factors such as the cytokinin receptors and the ligand specificity of receptors, and media conditions. Therefore, a special mechanism is required to control the cytokinin-responsive genes and the plant growth at specific concentration (Lomin *et al*., 2015). Sujatha and Kumari, (2007) reported that increasing the concentrations of cytokinin resulted in reducing the number of the microshoots of *A. vulgaris*. Catapan *et al.* (2000) reported that an average of 21 - 23 shoots could be induced from each nodal segment of *Phyllanthus caroliniensis* using MS medium supplemented with 0.5 – 1.0 mg/L 2iP. While, the lower concentration (0.25 mg/L) of 2iP failed to induce multiple shoots of *Psoralea corylifolia* L. in solid MS medium (Baskaran and Jayabalan, 2008).

Table 2. Effect of different cytokinins concentrations with 0.1 mg L^{-1} NAA, on the number of shoots, shoot length, callus width and length of *in vitro* grown *Artemisia herba-alba*

Cytokinin concentration mg/L	Shoot No.	Shoot Length (cm)	Callus Width (cm)	Callus length (cm)
		BA		
0.0	7.30 c*	2.50 a	1.30 b	1.20 c
0.5	10.50 b	1.71 b	1.49 b	1.56 b
1.0	16.4 a	1.70 b	1.92 a	1.30 c
1.5	7.50 c	1.39 c	1.87 a	1.90 a
2.0	11.6 b	1.34 c	0.88 c	0.81d
		Kinetin		
0.0	7.30 b*	2.50 a	1.30 b	1.20 a
0.5	8.20 a	1.19 bc	1.40 a	0.62 b
1.0	3.10 c	1.08 c	0.00 c	0.00 c
1.5	2.10 d	1.18 bc	0.00 c	0.00 c
2.0	3.50 c	1.29 b	0.00 c	0.00 c
		2 iP		
0.0	7.30 b*	2.50 a	1.30 c	1.20 c
0.5	8.80 b	2.02 b	2.08 a	1.86 a
1.0	22.9 a	2.61 a	1.45 bc	1.14 c
1.5	6.20 d	1.51 c	1.52 b	1.48 b
2.0	8.20 bc	1.57 c	0.46 d	0.52 d

* Means within columns for each cytokinin followed by the same letter (s) are not significantly different using LSD at 0.05.

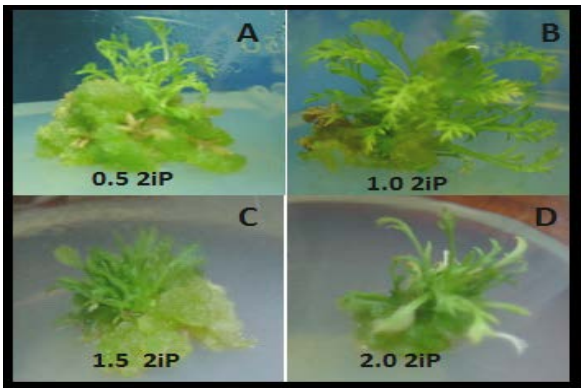

Figure 2. Effect of 2iP at different concentrations (A: 0.5, B: 1.0, C: 1.5 and D: 2.0 mg/L) on the microshoot proliferation of *A. herba-alba*.

Using GA_3 at 1.0 mg/L concentration gave the highest shoot numbers (14.2). While, the highest shoot length (3.41 cm) was obtained using GA_3 at a concentration of 1.5 mg/L (Table 3, Figure 3). Similar results were reported by Sujatha and Kumari (2007), who found that adding GA_3 to the media stimulated the shoot elongation of *A. vulgaris* L. The addition of GA_3 with BA or 6- (dimethyl-lallylamino)-purine (2iP) gave a similar response in Betula cultures (Jamison and Renfroe, 1998). GA_3 stimulates the elongation of shoots by inhibiting the action of auxins in meristematic regions (Taiz and Zeiger, 2014). Tian *et al.*, (2010), reported that GA_3 was beneficial for shoot and stem elongation.

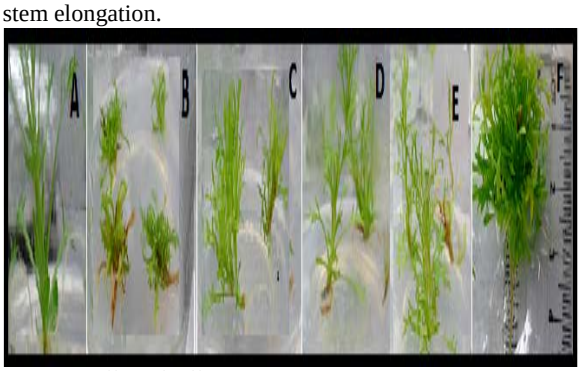

Figure 3. Effect of different GA_3 concentrations on *in vitro Artemisia herba-alba* grown on MS media (A): MS free medium, (B): 0.5 mg/L GA_3, (C): 1.0 mg/L GA_3, (D): 1.5 mg/L GA_3, (E): 2.0 mg/L GA3, (F): 3.0 mg/L GA_3.

Table 3. Effect of different GA_3 concentrations on the number of shoots, and shoot length of *in vitro* grown *Artemisia herba-alba*

GA_3 (mg/L)	Number of shoots	Shoot length (cm)
0.0	2.7 e*	1.45 e
0.5	4.0 d	1.98 d
1.0	14.2 a	2.50 c
1.5	7.5 b	3.41 a
2.0	7.0 b	2.50 c
3.0	5.8 c	2.90 b

* Means within each column followed by the same letter (s) are not significantly different using LSD at 0.05.

Adding GA_3 to the BA medium significantly increased the shoots length and number (Table 4). Combining GA_3 at (1.5 mg/L) with BA (0.5 mg/L) was effective in stimulating the shoot elongation (1.98 cm) of *A. herba-alba*. Similar results were obtained of the microshoots of *A. vulgaris L.* ; they were best elongated on MS medium containing 0.1 mg/L BA and 0.5 mg/L GA_3 (Sujatha and Kumari, 2007), and on Betula uber (Ashe) Fernald cultures (Jamison and Renfroe, 1998).

In the current study, supplementing the medium of GA_3 (1.5 mg/L) with different concentrations of 2iP increased the shooting length and number. The addition of (1.5 mg/L) GA_3 with (1.0 mg/L) 2iP effectively increased the shoot elongation (3.28 cm), and at the same time retained the highest number of shoots (23.6); (Table 4 and Figure 4). Similar results were reported for *Betula uber* (Ashe) Fernald, (Jamison and Renfroe, 1998).

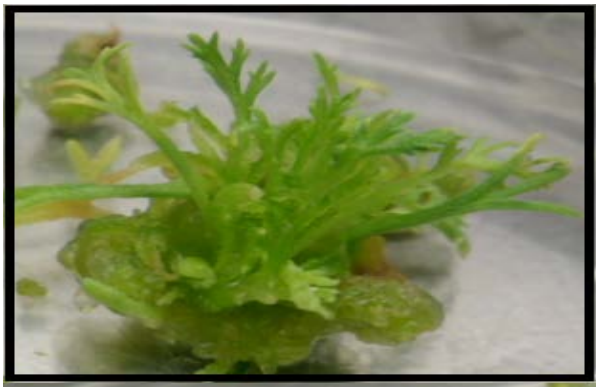

Figure 4. Effect of 1.5 mg/L GA_3 with 1.0 mg/L 2iP on *in vitro* grown *Artemisia herba-alba*.

Table 4. Effect of different cytokinins with concentrations plus 1.5 mg/L GA_3, on the number of shoots, shoot length and callus diameter of *in vitro* grown *Artemisia herba-alba*

Cytokinin (mg/L)	Number of shoots	Shoot length (cm)	Callus Diameter (cm)
BA			
0.0	8.1 d*	1.67 b	0.0 c
0.5	19.1 a	1.98 a	0.1 c
1.0	17.3 b	1.68 b	0.5 b
1.5	12.1 c	1.18 c	0.0 c
2.0	7.0 d	1.10 c	0.9 a
2ip			
0.0	8.1 d*	1.67c	0.0 c
0.5	16.6 b	2.90 b	0.0 c
1.0	23.6 a	3.28 a	0.0 c
1.5	15.2 bc	1.83 c	0.30 b
2.0	14.5 c	1.75 c	1.10 a
Zeatin			
0.0	8.1 d*	1.67 c	0.0 d
0.5	11.6 d	2.12 a	1.0 c
1.0	18.3 c	1.86 b	1.4 b
1.5	25.1 b	1.65 c	2.2 a
2.0	29.4 a	1.43 d	2.5 a

* Means within each column for each cytokinen followed by the same letter (s) are not significantly different using LSD at 0.05.

In the presence of GA_3, increasing the Zeatin concentration increased both the shoots' number and callus formation but with varied responses to shoot length (Table 4). Zeatin at 0.5 mg/L with 1.5 mg/L GA_3 gave the longest shoot (2.12 cm) with (11.6) shoot number and with less formation of callus. Among the different types of cytokinin tested, Pena-Ramirez *et al*., (2010), found that Zeatin was the most effective. However, a comparison of the relative effectiveness of different cytokinins for multiple shoot formation, Sharief and Chandra, (1991) reported that the most effective cytokinins in the order of their efficiency are: BA, Kinetin, Zeatin, and finally Adenine.

Among the different types of cytokinins (zeatin, BA, and 2iP) tested in this study, BA was the best. The explants cultured on BA medium performed healthy and strong plants. While using zeatin and 2iP resulted in some changes in the shape of leaves and the condensing of shoots (clusters).

3.2. In vitro Rooting

IBA gave the best root formation (root number and length) of *A. herba-alba in vitro* propagated plants (Table 5, Figure 5). *A. herba-alba* plants propagated at media supplemented with either IAA or NAA did not give any root. The maximum number of roots (18.8) resulted from the medium supplemented with 0.5 mg/L of IBA (Table 5). Root induction in *Teucrium stocksianum* Boiss was achieved on half-strength MS medium containing IBA (Bouhouche and Ksiksi, 2007). Similarly, *Coffee Arabica* microshoots were rooted on half MS medium supplemented with 3 mg/L of IBA (Ebrahim *et al.,* 2007). Also in *Talinum portulacifolium* L., roots development was facilitated by an MS medium supplemented with both 0.8 mg/L IBA and 0.2 mg/L NAA (Thangavel *et al.,* 2008). For *in vitro* grown *Origanium vulgare*, the highest number of roots was obtained at 1.6 mg/L IBA (Arafeh *et al*, 2006). Also in *Sealvia fruticosa* Mill, rooting was optimized at 0.6 mg/L IBA or 0.5 mg/L IAA (Arikat, 2004).

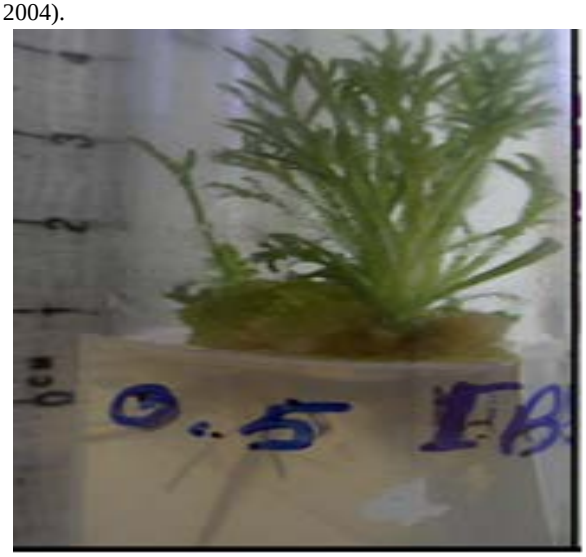

Figure 5. Effect of IBA at 0.5 mg/L on *in vitro* rooting of *Artemisia herba-alba* after 4 weeks.

Table 5. Effect of different IBA concentrations on shoot height, number of roots, root length and callus width of *in vitro* grown *Artemisia herba-alba*

IBA (mg/L)	Shoot length (cm)	No. of Roots	Root Length (cm)	Callus width (cm)
0.0	13.27 a*	12.70 b	8.22 b	1.00 c
0.5	11.57 b	18.8 a	14.08 a	2.80 a
1.0	1.72 cd	0.20 c	0.05 c	2.60 a
1.5	2.02 c	0.00 c	0.00 d	2.20 b
2.0	2.02 c	0.00 c	0.00 d	2.00 b

* Means within columns for each cytokinin followed by the same letter (s) are not significantly different using LSD at 0.05.

The current study concludes that the complexity and nonlinearity of spatiotemporal interactions between both cytokinin and auxins hormones and gene expression in root and shoot development, need modelling plant hormone gradients with a system approach in which experimental data and modelling analysis are closely combined.

3.3. Acclimatization

In vitro rooted plants of *A. herba-alba* showed a survival percentage of 50 %. Acclimatized plants appeared normal and did not exhibit any morphological abnormalities (Figure 6). The survival rate obtained in this study is lower than other micropropagated medicinal plants like *Origanium vulgare* 71 %, (Arafeh *et al.*, 2006), or *Capparis spinosa* 63 %, (Mussallam *et al.*, 2011). Some factors should be evaluated to improve the acclimatization stage for getting high plantlet survival rates.

Figure 6. Acclimatization of *Artemisia herba-alba*.

4. Conclusions

It can be concluded from this study that the seeds of *A. herba-alba* are considered a good starting material for establishing *in vitro* cultures due to the high percentage of *in vitro* germination. MS medium supplemented with plant growth regulators (0.5 mg/l BA + 1.0 mg/L GA_3) is considered suitable for mother stock multiplication. While 2iP 1.0 mg/L and BAP 1.0 mg/L gave higher shoot proliferation, satisfactory rooting was achieved only with IBA at 0.5 mg/L. Acclimatization of *A. herba-alba* resulted in (50 %) of acclimatized plants that remained healthy and showed normal growth in the greenhouse.

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Identification of the Bioactive Constituents and the Antibacterial, Antifungal and Cytotoxic Activities of Different Fractions from *Cestrum nocturnum* L.

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Abstract

This study was carried out to evaluate the antibacterial, antifungal and cytotoxic effects of chloroform, ethyl acetate and aqueous fractions obtained from a methanol 80 % extract of *Cestrum nocturnum* L. (family Solanaceae) aerial parts, and to investigate the bioactive constituents present in these fractions. The results showed that the chloroform fraction had the highest antibacterial effect and cytotoxicity against lung, colon and hepatocellular carcinoma cell lines, while the ethyl acetate fraction displayed the highest antifungal activity and cytotoxicity against cervical carcinoma cell lines. Phytochemical analysis showed the presence of triterpenes, coumarins and flavonoids in the chloroform fraction. Flavonoids, triterpenes and carbohydrates in the ethyl acetate fraction while tannins, flavonoids, saponins, carbohydrates and triterpenes were detected in the aqueous fraction. *β*-sitosterol, stigmasterol, protocatechuic acid and apigenin were isolated from the chloroform fraction; Kaempferol 8-*O*-methyl ether, kaempferol, kaempferol 3-*O*-α-rhamnoside and luteolin 7-*O*-*β*-glucoside were isolated from the ethyl acetate fraction, while kaempferol di-sugars: kaempferol 3-*O*-*β*-glucoside-7-*O*-α-rhamnoside and kaempferol 3,7-di-*O*-α-rhamnoside, were isolated from the aqueous fraction. This study proves the bioactivities of *C. nocturnum* aerial parts that can be explored as a source of medicinal compounds.

Keywords: *Cestrum nocturnum* L.; Aerial parts; Antimicrobial activity; Cytotoxicity; Phytochemical constituents.

1. Introduction

There is an urgent need to discover new antimicrobial and antitumor agents following the discovery of many infectious diseases (Hawkey, 2008; Jemal *et al*., 2011). Phytochemical screening of various plants revealed that secondary metabolites might be bioactive compounds with different properties such as antibacterial, antifungal, antioxidant, antidiabetic and anticancer (Gurib-Fakim, 2006; Phillipson, 2007; Alonso-Castro *et al.*, 2011). Therefore, compounds or extracts from the plant origin should be explored for the development of new formulations against different diseases.

Cestrum nocturnum L. is an evergreen shrub from the family Solanaceae that grows in tropical and sub-tropical regions throughout the world (Roig, 1988). Leaves of *C. nocturnum* were used to treat burns and swellings (Perez-Saad and Buznego, 2008). The volatile oil was used to prevent malaria (Mimaki *et al*., 2006). Leaves had significant analgesic and bactericidal effects (Huang *et al*., 2006). The inhibitory effects on the central nerve system and cardiac arrhythmic effect of this plant are also documented (Zeng *et al*., 2002). Polysaccharides extracts of *C. nocturnum* had antitumor effects (Zhong *et al*., 2008). Also the whole plant showed an antimicrobial activity against pathogenic microorganisms (Khan *et al.*, 2011). The essential oil from the plant showed an inhibition of some plant pathogens (Al-Reza *et al*., 2009a), and inhibitory effects on food-borne pathogens (Al-Reza *et al.*, 2009b). Its chloroform and toluene extracts gave behavior and analgesic effects (Rodriguez *et al*., 2005). Moreover, *C. nocturnum* leaves had calcinogenic glycoside (Mello, 2003), nocturnoside A and nocturnoside B (Ahmad *et al*., 1995), phenol glucosides (Sahai *et al*., 1994), flavonol glycosides and steroidal saponins (Mimaki *et al*., 2001; Mimaki *et al.*, 2002) and also some recent studies have been done on the plant (Doshi and Mukadam 2016, Chaskar *et al*. 2017). In the present study, the antibacterial, antifungal and cytotoxic activities of different fractions obtained from *C. nocturnum* aerial parts were evaluated. Furthermore, a phytochemical characterization of each fraction was carried out in order to

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understand the compounds involved in each individual fraction bioactivity.

2. Material and Methods

2.1. Equipments, Materials and Chemicals

UV/VIS: Shimadzu UV-visible recording spectrophotometer model-UV 240 (NRC, Egypt). Spectroscopic data (^{1}H-NMR, ^{13}C-NMR): Varian Unity Inova. MS (Finnigan MAT SSQ 7000, 70 ev). Silica gel (60-200 mesh, Merck) for column chromatography, Thin Layer Chromatography (TLC): pre-coated sheets of silica gel 60 F_{254} (Merck). Polyamide powder of caprolactam type (MN-polyamide SC6, Macherey Nagel, Pharmacia Fine Chemicals) for column chromatography, Paper Chromatography (PC), Whatman No.1 (Whatman Led. Maid Stone, Kent, England) sheets. Sephadex LH-20 (Sigma). Solvent mixtures, BAW (n-butanol: acetic acid: water 4:1:5 upper phase, 15% acetic acid: water: glacial acetic acid: 85:15).

2.2. Plant Material

Cestrum nocturnum aerial parts were collected from the Agricultural Research Centre, Giza, Egypt in May 2011 during flowering and identified by Dr. Mohammed El-Gebaly, Department of Botany, National Research Centre (NRC) and by Mrs. Tereez Labib, Consultant of Plant Taxonomy at the Ministry of Agriculture and director of Orman botanical garden, Giza, Egypt. A voucher specimen is deposited in the herbarium of Agricultural Research Centre, Giza, Egypt.

2.3. Preparation of the Plant Extracts

Air-dried powder from the aerial parts of *C. nocturnum* (680 g) was extracted with methanol 80 % several times at room temperature. The extract was concentrated to dryness by rotary evaporator giving 35 g of the crude extract. The extract was fractionated with chloroform, ethyl acetate solvents. The solvent of each partition was evaporated to dryness using rotary evaporator resulting in products weighing 9.5 g, 7 g and 16.5 g. of chloroform, ethyl acetate and water extraction, respectively. Each fraction was tested for the presence of bioactive compounds by using the following standard tests: Molisch test for carbohydrates, Shinoda test for flavonoids, Forth test for saponins, Salkowski for terpenes and sterols, $FeCl_3$ and Mayer's reagents for the detection of tannins and alkaloids, respectively (Harborne, 1973; Trease and Evans, 1989; Sofowra, 1993).

2.4. Antibacterial Activity

The following Gram (-) (*Enterobacter cloacae* human isolate, *Escherichia coli* ATCC 35210, *Pseudomonas aeruginosa* ATCC 27853 and *Salmonella typhimurium* ATCC 13311) as well as Gram (+) bacteria (*Bacillus cereus* clinical isolate, *Listeria monocytogenes* NCTC 7973, *Micrococcus flavus* ATCC 10240 and *Staphylococcus aureus* ATCC 6538) were used. These bacteria were obtained from Mycological Laboratory, Department of Plant Physiology, Institute for Biological Research "Siniša Stanković", University of Belgrade, Serbia. The antibacterial assay was carried out by microdilution method (Hanel and Raether, 1988; Espinel-Ingroff, 2001). The bacterial suspensions were adjusted with sterile saline to a concentration of 1.0×10^5 CFU/mL. The inocula were prepared daily and stored at 4°C until use. Dilutions of the inocula were cultured on solid medium to verify the absence of contamination and to check the validity of the inoculum. The minimum inhibitory and bactericidal concentrations (MICs and MBCs) were determined using 96-well microtiter plates. The bacterial suspension was adjusted with sterile saline to a concentration of 1.0×10^5 CFU/mL. The fractions of the same samples (three samples repeated three times) and three extract samples were collected for testing (1 and 10 mg/mL) in broth.

Tryptic Soy Broth (TSB) medium (100 µL) with bacterial inoculum (1.0×10^4 CFU per well) to achieve the wanted concentrations. The microplates were incubated on rotary shaker (160 rpm) for twenty-four hours at 37°C. The following day, 30 µL of 0.2 mg/mL solution of INT (*p*-iodonitrotetrazolium violet) were added, and the plates were returned to the incubator for at least one-half hour to ensure an adequate color reaction. Inhibition of growth was indicated by a clear solution or a definite decrease in color reaction. The lowest concentrations without visible growth (under the binocular microscope) were defined as concentrations that completely inhibited bacterial growth (MICs). The MBCs were determined by serial sub-cultivation of 2 µL into microtiter plates containing 100 µL of broth per well and further incubation for twenty-four hours. The lowest concentration with no visible growth was defined as the MBC, indicating 99.5 % killing of the original inoculum. The optical density of each well was measured at a wavelength of 655 nm by microplate manager 4.0 (Bio-Rad Laboratories), and was compared with a blank and the positive control. The antibiotics streptomycin and ampicillin were used as positive controls (1 mg/mL in sterile physiological saline). Three independent experiments were performed in triplicate.

2.5. Antifungal Activity

The used fungi; *Aspergillus fumigatus* (ATCC 1022), *Aspergillus versicolor* (ATCC 11730), *Aspergillus ochraceus* (ATCC 12066), *Aspergillus niger* (ATCC 6275), *Trichoderma viride* (IAM 5061), *Penicillium funiculosum* (ATCC 36839), *Penicillium ochrochloron* (ATCC 9112) and *Penicillium verrucosum* var. *cyclopium* were obtained from Mycology Laboratory, Department of Plant Physiology, Institute for Biological Research "Siniša Stanković", University of Belgrade, Serbia. The Micromycetes were maintained on malt agar and the cultures were stored at 4° C and sub-cultured once a month. The antifungal assay was carried out by a modified microdilution technique (Hanel and Raether, 1988; Espinel-Ingroff, 2001). The fungal spores were washed from the surface of agar plates with sterile 0.85 % saline containing 0.1 % Tween 80 (v/v). The spore suspension was adjusted with sterile saline to a concentration of approximately 1.0×10^5 in a final volume of 100 µL per well. The inocula were stored at 4° C for further use. Dilutions of the inoculum were cultured on solid malt agar to verify the absence of contamination and to check the validity of the inoculum. MIC determinations were performed by a serial dilution technique using 96-well microtiter plates. The examined fractions of the same sample (repeated three times) were diluted in 5 % of

DMSO (1 mg/mL and 10 mg/mL) and added in broth malt medium (MA) with inoculum. The microplates were incubated in a rotary shaker (160 rpm) for seventy-two hours at 28° C. The lowest concentrations without visible growth (at the binocular microscope) were defined as MICs. The fungicidal concentrations (MFCs) were determined by serial subcultivation of 2 µL of tested fractions dissolved in medium and inoculated for seventy-two hours, into microtiter plates containing 100 µL of broth per well and further incubation for seventy-two hours at 28° C. The lowest concentration with no visible growth was defined as MFC indicating 99.5 % killing of the original inoculum. The fungicides bifonazole and ketoconazole were used as positive controls (1-3500 µg/mL). Three independent experiments were performed in duplicate.

2.6. Cytotoxicity in Human Tumor Cell Lines and in Liver Primary Cell Culture

Five human tumor cell lines were used: MCF-7 (breast adenocarcinoma), NCI-H460 (non-small cell lung cancer), HCT-15 (colon carcinoma), HeLa (cervical carcinoma) and HepG2 (hepatocellular carcinoma). Cells were routinely maintained as adherent cell cultures in RPMI-1640 medium containing 10 % heat-inactivated fetal bovine serum (FBS) and 2 mM glutamine (MCF-7, NCI-H460 and HCT-15) or in DMEM supplemented with 10 % FBS, 2 mM glutamine, 100 U/mL penicillin and 100 mg/mL streptomycin (HeLa and HepG2 cells), at 37 ºC, in a humidified air incubator containing 5 % CO_2. Each cell line was plated at an appropriate density (7.5×10^3 cells/well for MCF-7, NCI-H460 and HCT-15 or 1.0×10^4 cells/well for HeLa and HepG2) in 96-well plates. Sulforhodamine B assay was performed according to a procedure previously described by Guimarães *et al*., (2013).

For the hepatotoxicity evaluation, a cell culture was prepared from a freshly-harvested porcine liver obtained from a local slaughter house, according to a procedure established by Guimarães *et al*. (2013); it was designed as PLP2. Cultivation of the cells was continued with direct monitoring every two to three days using a phase contrast microscope. Before confluence was reached, cells were subcultured and plated in 96-well plates at a density of 1.0×10^4 cells/well, and cultivated in DMEM medium with 10 % FBS, 100 U/mL penicillin and 100 µg/mL streptomycin. Three independent experiments were performed in triplicate, and the results were expressed as mean values ± standard deviation (SD).

2.7. Phytochemical Characterization of C. nocturnum Fractions

2.7.1. Phytochemical Analysis of Chloroform Fraction

It was subjected to silica gel column chromatography using chloroform ($CHCl_3$) as eluent and a gradually increasing amount of methanol (MeOH). Four compounds were isolated. Compound one (see figure 3) isolated from $CHCl_3$:MeOH (98:2) elution, and compound two was isolated through elution with $CHCl_3$:MeOH (96:4). Compound three was isolated through elution with $CHCl_3$: MeOH (90:10), and then compound four was isolated through elution with $CHCl_3$: MeOH (85:15).

2.7.2. Phytochemical Analysis of Ethyl Acetate Fraction

It was subjected to silica gel column chromatography using $CHCl_3$ as eluent and a gradually increasing amount of ethyl acetate (EtOAc) and MeOH. Compound five was isolated from $CHCl_3$: EtOAc (50:50) elution, compound six was isolated from EtOAc elution and compounds seven and eight were isolated through further elution with EtOAc and MeOH, gradually.

2.7.3. Phytochemical Analysis of Aqueous Fraction

It was subjected to polyamide column chromatography using distilled water and gradually increasing amounts of MeOH. Compounds nine and ten were isolated through this elution. All the compounds were purified on sephadex LH–20 column which was eluted with mixtures of methanol with distilled water.

2.7.4. Acid Hydrolysis of Flavonoid Glycosides

Solutions of 5 mg of compounds 7, 8, 9 and 10 in 5 ml 10% HCl were heated for five hours. The aglycones were extracted with EtOAc and identified by co-TLC with authentic standards. The sugars in the aqueous layer was identified by co-paper chromatography (co-PC) with authentic markers on Whatman No. 1 sheets in solvent system (*n*-BuOH-AcOH-H_2O 4:1:5 upper layer).

3. Results and Discussion

The obtained results for the antibacterial and antifungal activity are presented in Tables 1 and 2, Figures 1 and 2, respectively. The chloroform fraction showed activity against all the tested bacteria (MIC 0.6-2.5 mg/mL and MBC 1.25-5.0 mg/mL); ethyl acetate fraction exhibited MICs of 1.25-3.75 mg/mL and MBCs of 2.5-7.5 mg/mL, and the aqueous fraction revealed MICs of 0.6-1.25 mg/mL and MBCs of 1.25-5.0 mg/mL. It can be observed that the chloroform fraction gave the highest antibacterial activity, except for *L. monocytogenes* and *E. cloacae*, where the aqueous fraction showed a higher effect. The least effective was the ethyl acetate fraction. The most sensitive bacteria were *E. cloacae*, while the most resistant one was *P. aeruginosa*, followed by *L. monocytogenes*.

All the fractions were more active against microfungi (Table 2 and Figure 2). The chloroform fraction inhibited all microfungi at 0.15-0.6 mg/mL and completely stopped the growth at 0.3-1.25 mg/mL. The ethyl acetate fraction showed inhibitory concentrations at 0.075-0.3 mg/mL and fungicidal effects at 0.15-0.6 mg/mL. The aqueous extract showed MICs of 0.15-2.5 mg/mL and MFCs at 0.60-5.0 mg/mL. The highest activity was observed for the ethyl acetate fraction, while the aqueous fraction showed the lowest antifungal effect. The most sensitive microfungi was *P. ochrochloron*, on the other hand, *A. fumigatus* was the most resistant to the tested fractions. The ethyl acetate fraction showed higher antifungal effect towards *P. ochrochloron* than both mycotics. All the tested fractions exhibited higher antifungal activity against *P. funiculosum* than ketoconazole. Chloroform and ethyl acetate fractions possessed higher effect on *P. verrucosum* var. *cyclopium* than ketoconazole. The Chloroform extract showed the same MIC but higher MFC for *A. niger* and *P. ochrochloron* than ketoconazole. In general, bacteria are more resistant than fungi (Soković et al., 2010). The

observed antibacterial activity from the chloroform fraction can be attributed to the presence of compounds; stigmasterol isolated from that fraction has a moderate antibacterial effect when compared to the reference drug (ciprofloxacin). This confirmed the antibacterial activity of stigmasterol against *Acetobacter* sp., *E. coli*, *S. aureus*, *Streptococcus* sp., and *P. aeruginosa* (Sileshi *et al.*, 2012); *β*-sitosterol also has a moderate antibacterial effect (Sileshi *et al*., 2012). Apigenin has a good antibacterial effect against several bacterial strains and the values are close to the antibacterial property of broad-spectrum, antibiotic tetracycline (Rui *et al.*, 2013). Also, only chloroform fraction possessed coumarins which can have influence in its higher antibacterial effect. It has been reported that some groups in coumarins skeleton result in increasing lipophilicity of the molecule (Stavri and Gibbons, 2005).

Table 1. Antibacterial activity of different fractions of *Cestrum nocturnum* aerial parts.

Bacteria	Chloroform MIC (mg/mL)* MBC (mg/mL)	Ethyl acetate MIC (mg/mL) MBC (mg/mL)	Aqueous MIC (mg/mL) MBC (mg/mL)	Streptomycin MIC (mg/mL) MBC (mg/mL)	Ampicillin MIC (mg/mL) MBC (mg/mL)
Bacillus	0.6±0.06	1.25±0.10	1.25±0.00	0.05±0.005	0.1±0.05
cereus	1.25±0.10	5.0±0.00	2.5±0.30	0.1±0.03	0.15±0.05
Micrococcus flavus	0.6±0.00	1.25±0.10	1.25±0.00	0.125±0.015	0.1±0.00
	2.5±0.30	5.0±0.60	2.5±0.00	0.25±0.003	0.15±0.06
Staphylococcus aureus	0.6±0.06	2.5±0.30	1.25±0.10	0.25±0.003	0.1±0.05
	2.5±0.00	5.0±0.30	2.5±0.30	0.50±0.00	0.15±0.00
Listeria monocytogenes	2.5±0.30	2.5±0.00	1.25±0.10	0.15±0.05	0.15±0.03
	5.0±0.60	5.0±0.00	5.0±0.60	0.30±0.03	0.30±0.01
Escherichia coli	0.6±0.00	1.25±0.10	1.25±0.10	0.10±0.03	0.30±0.05
	2.5±0.30	2.5±0.30	2.5±0.30	0.50±0.06	0.50±0.06
Pseudomonas aeruginosa	1.25±0.10	3.75±0.10	1.25±0.00	0.05±0.006	0.10±0.00
	2.5±0.30	7.5±0.00	2.5±0.30	0.10±0.0	0.20±0.05
Enterobacter cloacae	0.6±0.06	1.25±0.10	0.6±0.60	0.05±0.005	0.15±0.00
	2.5±0.30	5.0±0.60	1.25±0.00	0.10±0.06	0.20±0.06
Salmonella typhimurium	1.25±0.00	1.25±0.00	1.25±0.00	0.05±0.006	0.15±0.03
	2.5±0.30	5.0±0.30	2.5±0.30	0.10±0.03	0.20±0.00

* Minimum inhibitory concentration - MIC; Minimum bactericidal concentration - MBC.

Table 2. Antifungal activity of different fractions of *Cestrum nocturnum* aerial parts.

Fungi	Chloroform MIC (mg/mL)* MBC (mg/mL)	Ethyl acetate MIC (mg/mL) MBC (mg/mL)	Aqueous MIC (mg/mL) MBC (mg/mL)	Bifonazole MIC (mg/mL) MBC (mg/mL)	Ketoconazole MIC (mg/mL) MBC (mg/mL)
Aspergillus	0.6±0.06	0.3±0.00	2.5±0.00	0.15±0.03	0.20±0.03
fumigatus	1.25±0.10	0.6±0.06	5.0±0.60	0.20±0.06	0.50±0.05
Aspergillus	0.6±0.00	0.3±0.06	0.6±0.06	0.10±0.05	0.20±0.03
versicolor	1.25±0.00	0.6±0.00	2.5±0.30	0.20±0.03	0.50±0.05
Aspergillus	0.3±0.06	0.3±0.06	0.6±0.06	0.15±0.03	0.15±0.03
ochraceus	0.6±0.06	0.6±0.00	1.25±0.00	0.20±0.06	0.20±0.06
Aspergillus	0.2±0.03	0.2±0.03	0.6±0.06	0.15±0.03	0.20±0.06
niger	0.3±0.00	0.6±0.06	1.25±0.10	0.20±0.03	0.50±0.05
Penicillium verrucosum	0.2±0.03	0.2±0.03	0.6±0.06	0.15±0.05	1.0±0.30
	0.3±0.03	0.6±0.06	1.25±0.00	0.20±0.03	1.0±0.30
Penicillium	0.15±0.03	0.075±0.00	0.15±0.03	0.20±0.06	0.20±0.03
ochrochloron	0.3±0.06	0.15±0.03	0.6±0.06	0.25±0.05	0.50±0.06
Penicillium	0.3±0.00	0.3±0.06	0.15±0.00	0.20±0.03	2.5±0.30
funiculosum	0.6±0.00	0.6±0.00	0.6±0.06	0.25±0.05	3.5±0.50
Trichoderma	0.6±0.06	0.3±0.06	0.15±0.00	0.10±0.05	0.20±0.03
viride	1.25±0.00	0.6±0.00	0.6±0.06	0.20±0.06	0.30±0.06

* Minimum inhibitory concentration - MIC; Minimum fungicidal concentration - MFC.

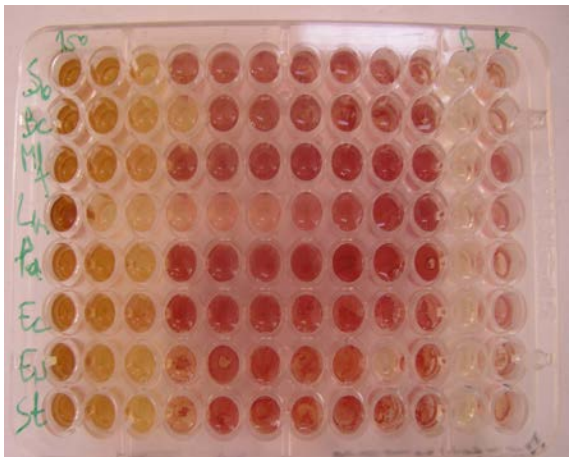

Figure 1. Antibacterial activity of the chloroform extract of *Cestrum nocturnum* aerial parts by microdilution method. Antibacterial effect of extract at different concentrations 0.6-2.5 mg/mL (MIC and MBC). Comparison between control-bacterial growth (K-red color) and treated samples (yellow color MIC and MBC) and treated samples with no activity (red color) in INT assay.

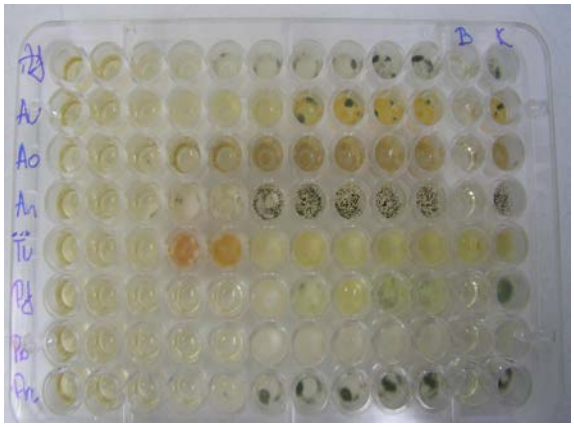

Figure 2. Antifungal activity of the chloroform extract of *Cestrum nocturnum* aerial parts by microdilution method. Control (K-fungal growth) and treated samples (MIC and MFC, 0.2-1.25 mg/mL–clean, transparent wells with no fungal growth, rows 1-3) and treated samples with fungal growth and no activity, rows 4-10.

Kaempferol isolated from ethyl acetate fraction showed a significant antimicrobial activity against all the tested organisms with MIC values between 16 and 63 µg/mL (Teffo *et al.*, 2010). Kaempferol di-sugars isolated from aqueous fraction showed a good antimicrobial activity against most of the studied microorganisms (Salwa *et al.*, 2012).

The three fractions revealed cytotoxicity against different human tumor cell lines (Table 3). Nevertheless, the chloroform fraction proved to be more active against NCI-H460 (lung carcinoma; GI_{50} 70.54 µg/mL), HCT-15 (colon carcinoma; GI_{50} 86.65 µg/mL) and HepG2 (hepatocellular carcinoma, GI_{50} 171.83 µg/mL) cell lines, while the ethyl acetate and the aqueous fractions were more efficient against HeLa (cervical carcinoma, GI_{50} 57.48 µg/mL) and MCF-7 (breast carcinoma, GI_{50} 55.28 µg/mL) cell lines, respectively. The ethyl acetate fraction (up to 400 µg/mL) was not toxic to non tumor liver cells; the other two fractions showed some hepatotoxicity, but at higher concentrations than the ones allowing anti-tumor effects. Ellipticine, a positive control, was used as a standard for comparison with the studied fractions.

The Cytotoxic activity observed for the chloroform fraction can be attributed to the presence of apigenin which showed a significant cytotoxic effect against different tumor cell lines (Rui *et al.*, 2013). The observed cytotoxic activity of ethyl acetate fraction can be attributed to the presence of the main flavonoid constituent, luteolin 7-*O*-glucoside which showed significant cytotoxicity (LD_{50} = 85 ±34 μg/mL) (Rilka *et al*., 2003), while the observed cytotoxicity of the aqueous fraction can be attributed to the presence of kaempferol di-sugars. Many reports showed that kaempferol glycosides cause cell death in a variety of cancer cells (Brusselmans *et al.*, 2005, Tomczyk *et al*., 2008).

The results of phytochemical analysis of the fractions of *C. nocturnum* aerial parts from the methanol 80 % extract are included in Table 4. Triterpenes, coumarins and flavonoids were detected in the chloroform fraction. Flavonoids, triterpenes and carbohydrates were detected in the ethyl acetate fraction, while tannins, flavonoids, saponins, carbohydrates and triterpenes were detected in the aqueous fraction. Isolation and purification of chloroform, ethyl acetate and aqueous fractions allowed the identification of ten bioactive compounds: β-sitosterol, stigmasterol, protocatechuic acid and apigenin from chloroform fraction; kaempferol 8-*O*-methyl ether, kaempferol, kaempferol 3-*O*-α-rhamnoside and luteolin 7-*O*-β-glucoside from ethyl acetate fraction; and kaempferol-disugars, kaempferol 3-*O*-β-glucoside-7-O-α-rhamnoside and kaempferol 3,7-di-*O*-α-rhamnoside from aqueous fraction (Figure 3). The chemical structures were determined by UV, ^{1}H-NMR, ^{13}C-NMR, and MS analyses.

Table 3. Cytotoxicity of different fractions of *Cestrum nocturnum* aerial parts in human tumor cell lines and in non-tumor liver primary culture.

	Chloroform (GI_{50}, µg/mL)*	Ethyl acetate (GI_{50}, µg/mL)	Aqueous (GI_{50}, µg/mL)	Ellipticine (GI_{50}, µg/mL)
HCT-15 (colon carcinoma)	86.65±5.86	223.00±4.03	98.11±8.48	1.91±0.06
HepG2 (hepatocellular carcinoma)	171.83±11.85	213.18±9,50	225.86±16.92	3.22±0.67
HeLa (cervical carcinoma)	62.49±4.36	57.48±5.37	61.34±3.59	1.14±0.21
MCF-7 (breast carcinoma)	66.22±5.70	194.22±5.73	55.28±4.87	0.91±0.04
NCI-H460 (lung carcinoma)	70.54±6.56	185.27±5.18	83.41±0.91	1.42±0.00
PLP2 (non-tumour liver cells)	323.44±9.67	>400	285.44±6.03	2.06±0.03

* GI_{50} values correspond to the sample concentration achieving 50% of growth inhibition in human tumour cell lines or in liver primary culture PLP2.

Table 4. Phytochemical analysis of different fractions of *Cestrum nocturnum* aerial parts.

Chemical Constituents	Chloroform	Ethyl acetate	Aqueous
Carbohydrates and/or glycosides	- *	+	+
Tannins			
Condensed tannins	-	-	+
Hydrolysable tannins	-	-	+
Alkaloids and/or nitrogenous bases	-	-	-
Flavonoids	+	+	+
Sterols and/or triterpenes	+	+	+
Saponins	-	-	+
Coumarins	+	-	-

* (-) denotes the absence of the constituents; (+) denotes the presence of the constituents.

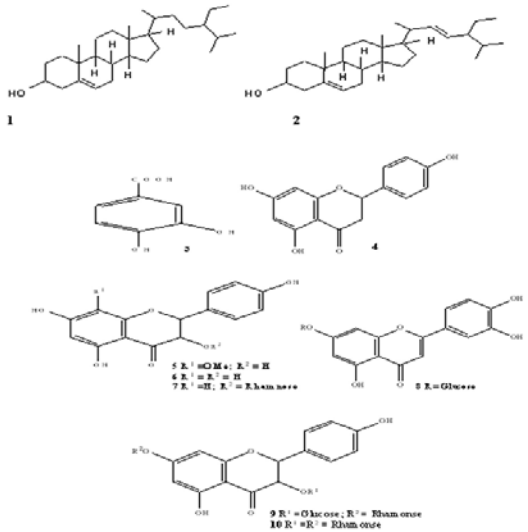

Figure 3. Chemical structure of the compounds isolated and identified in chloroform (1. *β*-sitosterol, 2. stigmasterol, 3. protocatechuic acid, 4. apigenin), ethyl acetate (5. kaempferol 8-*O*-methyl ether, 6. kaempferol, 7. kaempferol 3-*O*-α-rhamnoside, 8. luteolin 7-*O*-β-glucoside) and aqueous (9. kaempferol 3-*O*-β-glucoside-7-*O*-α-rhamnoside, 10. kaempferol 3,7-di-*O*-α-rhamnoside) fractions.

4. Conclusions

The present results proved that the chloroform fraction possessed the highest antibacterial activity due to the presence of coumarins and the presence of *β*-sitosterol, stigmasterol, protocatechuic acid and apigenin which could also contribute to the highest antibacterial activity of that fraction, and to the highest cytotoxicity against lung, colon and hepatocellular carcinoma cell lines. On the other hand, compounds such as kaempferol 8-*O*-methyl ether, kaempferol, kaempferol 3-*O*-α-rhamnoside and luteolin 7-*O*-*β*-glucoside could be related to the highest antifungal activity and cytotoxicity against cervical carcinoma cell line, displayed by the ethyl acetate fraction. Nevertheless, the different bioactivities exhibited by the fractions can also be due to the synergistic effect of different compounds present in the fractions or by the presence of other compounds that may be active even in small concentrations. The results obtained herein support the traditional medicinal use of *Cestrum nocturnum*, and provide grounds for further establishment of its use. Further studies are required to develop strategies for practical application.

Conflict of interest

There is no conflict of interest associated with the authors of this paper.

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Is Rh Positivity a Possible Risk Factor for Lung Cancer?

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Abstract

The association between ABO and Rh blood groups and the incidence of various types of cancers has been previously investigated, but with no definite consensus reached. Since lung cancer has become one of the most leading causes of cancer-related deaths, its relation to ABO/Rh is being investigated in many centers. To explore the relationship between ABO and Rh blood groups and the risk of lung cancer. A case control study was conducted to explore the relationship between blood groups/Rh factor and the risk of lung cancer. Lung cancer cases (n=458) were identified from two major university hospitals. Control subjects included a total of 3,847 patients in which none is a cancer patient. ABO and Rh blood groups were typed and compared between cases and controls. The adjusted effect of Rh, but not ABO blood group, was significantly associated with the risk of lung cancer. The results of this study showed that Rh positive subjects are more likely to have lung cancer than the Rh negative subjects, indicating an association between Rh positivity and the risk of lung cancer. The Rh factor showed a significant association with lung cancer, but not with ABO blood groups. Further studies are needed to investigate this association to determine the role of Rh in the development of lung cancer.

Key-words: Lung, Cancer, Blood group, Rh, Jordan

1. Introduction

Over the last few decades, lung cancer has become one of the major health issues and the leading cause of cancer-related deaths (Ferlay *et al*., 2015). With most cases being diagnosed at an advanced stage, improving the outcomes in these cases is hard, compared to the treatment of cases with an early diagnoses, which is considered an important prognostic factor (Finkelstein *et al*., 1986). Identifying the risk factors of lung cancer is critical for an early diagnosis and inclusion in the screening programs (Aberle *et al*., 2011).

Blood groups are believed to have functions in cell physiology and human pathology (Landsteiner, 1900; Mohandas and Narla, 2005). ABO antigens are expressed on the surface of cells other than red blood cells, like epithelial cells of urothelium, gastrointestinal, mucosa and lung (Zmijewski, 1978 ; Graziano *et al*., 1997). Alterations of these cell surface structures are thought to be important for tumor development (Dall'olio, 1996). ABO blood group genes are mapped at the 9q chromosome, and are of seven exons, in which many cancers might result if any genetic alteration happened (Hosoi, 2008).

Overall, the association between blood groups and the incidence of various types of cancers have been previously investigated. Blood groups O, or non-A, was reported to have a low incidence of cancer, while blood groups non-O, demonstrated a higher risk for cancer (Allouh *et al*. 2017). The association between ABO blood groups and the incidence of gastric and/or pancreatic cancer is considered to be reliable and convincing, as confirmed by large-scale meta-analyses (Wolpin *et al*., 2009; Amundadottir *et al*., 2009).

Despite doing many studies, the results and conclusions reported were not identical. Higher frequency of blood group A and lower frequency of blood group O were reported in pancreatic cancer (Allouh *et al*. 2017). Another study concluded that the non-O blood group individuals were reported to have a higher risk of developing pancreatic cancer, while the highest risk is found to be associated with blood group B individuals (Wolpin *et al.*, 2009). Single nucleotide polymorphism at the ABO gene

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locus was also found to have an important association with the risk of pancreatic cancer (Amundadottir *et al.*, 2009).

Utilizing the Scandinavian Donations and Transfusions database on more than a million healthy blood donors, it was reported that people with the blood group A had a higher risk of developing gastric cancer (Edgren *et al.*, 2010). On the other hand, no significant correlation between gastrointestinal stromal tumor and the blood group antigen and Rh were reported. (Ürün *et al.*, 2012).

The current study was conducted to assess the relationship between ABO blood grouping/Rh factor and lung cancer in Jordan.

2. Methods

An observational case control study was conducted to assess the relationship between ABO and Rh blood groups and the risk of developing lung cancer. Cases (n=458) were identified from the medical records of two major university hospitals. All patients with a confirmed diagnosis of a primary lung cancer between 2005 and 2015 were included in the study as cases. Control subjects included a total of 3,847 non-cancer patients identified from the laboratory within the same time frame. Medical records of all controls did not reveal any cancer diagnosis. The study was approved by the Institutional Review Board Committees of our institution.

ABO/RH typing for both cases and controls were determined by withdrawing samples of blood and using DG Gel ABO/RH, manufactured by: Diagnostic Grifols, Spain. To prepare the slides, the samples were embedded in paraffin then cooled using "Thermoscientific: Histostar", then they were cut into thin sections (2-3 micrometer) using "Sakura: Accu-Cut SRM 200 Rotary Microtome" Then they were bathed in warm water (36 degrees Celsius), and only the clear sections were picked and fixed on slides using saline. They were then stained with H&E using "Leica Autostainer XL". After the diagnosis was confirmed by H&E stain, further subtyping was determined by immunohistochemistry. The slides were first dewaxed, hydrated by ethanol and were then treated for Ag retrieval using PT. Link (DAKO, Denmark) and a high ph citrate buffer for twenty minutes. The slides were then cooled and blocked by 3 % of H2O2. Primary antibodies for (Ki67:IR626, SYNAPT: IR660, CK5/6: IR780, TTF1:IR056, CYT7:IR619, CK20: IR777) all from (DAKO. Denmark). Signal detection was made using flex -envision kit (k-8000 DAKO). The diagnosis and subtyping of lung cancer cases was then made by the Department of Pathology, and was later reviewed by three different consultants for research purposes.

After the cases and controls were identified utilizing the electronic medical records of the two hospitals, data was then extracted from the data warehouse in an EXCEL format. Statistical Package for Social Sciences (SPSS) (version 20) was used for data management and analysis. Chi-square test was utilized to assess the relationship between ABO/Rh blood group and lung cancer diagnosis. Unadjusted effect of variables under investigation was reported using Odds Ratios and 95 % Confidence Intervals (OR, 95% CI). Alpha level was set at 0.05.

3. Results

A total of 458 lung cancer cases and 3,847 controls were included in the current analysis. A statistically significant difference of Rh was detected between the lung cancer cases and controls. About 92 % of the lung cancer cases and 86.3 % of the controls were Rh positive ((OR [95% C.I.] =1.75 [1.23, 2.43]), P <0.001). The distribution of blood groups among the cases and controls was not statistically significant (P >0.05) (Table 1).

Table 1. Distribution of the Study Participants by Disease Status and Blood group and Rh.

Variable		Disease status					
		Case (N=458)		Control (N=3,847)			
		Number	%	Number	%	*P*-Value^	OR (95% C.I)
Blood Group	A	181	39.5%	1,505	39.1%	*P*>0.05	1.02 (0.83-1.25)
	AB	30	6.6%	229	6.0%		1.11 (0.72-1.65)
	B	90	19.7%	684	17.8%		1.13 (0.88-1.45)
	O	157	34.3%	1429	37.1%		1.10 (0.87 - 1.37)
Rh	Negative	38	8.3%	527	13.7%	0.0012	Reference
	Positive	420	91.7%	3320	86.3%		1.75 (1.23-2.43)

^ Chi-square test

4. Discussion

Lung cancer accounts for 13 % of all newly-diagnosed cancers. It is one of the most worldwide common cancers (Abdel-Razeq *et al*. 2015). In Jordan, the number of the new cancer cases diagnosed among Jordanians has increased by 44 % over the past decade, rising from 3,362 cases in 2000 to 4,849 in 2010. The current study was designed to explore the relationship between blood grouping/Rh and lung cancer among Jordanians. The researchers found that while Rh-positivity was associated with the increased risk of lung cancer, no statistical association was found between ABO blood groups and lung cancer.

The current study examined 458 lung cancer patients (Adenocarcinoma (n = 204), Squamous cell carcinoma (n = 126), poorly differentiated Non-small cell carcinoma (n = 43), Small cell carcinoma (n = 53), Adenosquamous, Large cell neuroendocrine, Salivary gland tumors of lung (n = 32). The researchers found that the percentage of Rh positivity in lung-cancer patients was higher than in the control group, suggesting that Rh positivity increases the risk of lung cancer compared to the Rh negativity. These findings are in agreement with data from (Oguz *et*

al.,2013), showing that there was no significant difference between the patients with lung cancer and the control group in terms of distribution of the ABO blood groups. Other data also found that no significant difference among the patients with small cell cancer and those with non-small cell cancer in terms of distribution of the ABO blood groups and Rh factor (Oguz *et al.*, 2013; Unal *et al.*, 2013). On the other hand, the findings of this study are not in agreement with previous studies conducted by Urun, Rummel and Kumar. (Urun *et al*., 2013; Rummel *et al*., 2016; Kumar *et al*., 2014). Urun (Urun *et al*., 2013**)** reported a significant relationship between the ABO blood types and lung cancer; and that the O blood group and Rh-positivity are associated with a 14 % and a13 % risk reduction of lung cancer, respectively. Rummel **(**Rummel *et al*., 2016) reported that when the tumors were sub-classified, there was an increased frequency of the A blood type and a decreased frequency of the O blood type in the patients with proximal tumors and vice versa with distal tumors. According to Fukumoto (Fukumoto *et al*., 2015) for lung cancer patients, the expression of blood group antigen A in tumor cells was reported to be a favorable prognostic factor. The multivariate survival analysis for Franchini (Franchini *et al*., 2016) showed the ABO blood group to be an independent prognostic factor in addition to age, sex, smoking status, p-stage, and serum CEA level. The blood group A antigen may have a negative effect on the prognosis of surgically-managed patients with NSCLC.

On the other hand, Kumar (Kumar *et al*., 2014) reported the absence of association between ABO blood grouping and lung cancer and that the Rh factor had a statistically significant two-fold effect on increasing the risk for NSCLC.

5. Conclusions

Patients with lung cancer were found to have a higher percentage of Rh positivity compared to the non-cancer patients in our population, which suggests an association between Rh positivity and the risk of lung cancer.

Since no proper screening program for lung cancer is well-developed yet, Rh positive patients with other risk factors should be considered for lung cancer invistigations earlier.

The relation between the type of Blood group/Rh and the incidence of lung cancer is not simple. It could be a multifactorial relationship. Other blood subtypes may play a role as confounding factors. It is advised to include other blood subtypes in future studies. The current study recommends further studying of the relation to define the mechanisms by which blood grouping and Rh factor may be related to the lung cancer cases, and even to other malignancies.

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The Relationship between Iodine Nutrition, Thyroid Function and Obstetrical Outcomes for Jordanian Pregnant Women

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Abstract

Iodine deficiency causes many health problems to pregnant women and their newborns. Knowledge about the impact of Iodine deficiency can improve health-related practices, such as seeking dietary and exogenous supplements to improve iodine levels. This study evaluates the knowledge level of pregnant women concerning the significance of the intake of iodine-supported salt and iodine-rich diets during pregnancy. It also investigates the impact of this knowledge on certain dietary habits and the effects of iodine deficiency on several maternal and child complications. It explores the impact of iodine deficiency on the health condition of mothers and their newborns. Among the 500 women surveyed in this study, the majority were young (aged<45) and had previous pregnancies and at least one abortion. This study found that women had limited knowledge and poor dietary supplements of iodine relevant to a relatively high prevalence of abortion and anemia. Iodine deficiency is associated with several health conditions, such as infertility, fetal deaths, post-partum hemorrhage, and learning difficulties in the children to be born. This study suggests developing national policies and programs to improve people's awareness regarding iodine-related health conditions.

Keywords: Iodine, Nutrition, Women, pregnancy, Jordan

1. Introduction

Iodine has a structural role forming the thyroid hormones, which influence many functions in the body (Rohner *et al*., 2014). It has been reported in the literature that even mild forms of iodine deficiency can be responsible for changes in the neurological and cognitive developments of the fetus and the young child (Bath *et al*., 2013). This impact can also extend to the school age developing cognitive and learning difficulties in the children as they grow up (Zimmerman and Boelaert, 2015). Furthermore, hypothyroidism and goiter, which are adaptive responses secondary to the low dietary intake of iodine (WHO, 2013), have been reported to increase the likelihood of iodine-related conditions later in the life, such as neurological deficits (Zimmerman and Andersson, 2012).

Iodine is an essential nutritional component during pregnancy for the health of women and their newborns (Pires *et al*., 2017). It is estimated that iodine requirements are increased to approximately 220 and 270 µg/day during pregnancy and lactation, respectively (Simpson *et al*., 2011). This change is related to the increased production of thyroxine by the mother to maintain the euthyroid state, transfers thyroid hormone to the fetus along with iodine, due to the increased renal iodine clearance by the mother (Glenoer, 2007). It has also been reported that the thyroid gland increases in size by 10 % in iodine replete-countries, and that this increase reaches 20 % to 40 % in areas of reported iodine deficiency during pregnancy in healthy women (Alexander *et al*., 2017). Consequently, thyroid hormones increase by nearly 50 % putting further demands on women to increase their uptake by a comparable percentage (Stagnaro-Green *et al*., 2011).

Among the most challenging issues concerning iodine deficiency, is the knowledge of the women about the key role of iodine for their health and the fetus development. Charlton *et al*. (2012) argued that poor knowledge about iodine is a main contributor to hypothyroidism among pregnant women. Unlike most other essential nutrients, iodine status is linked mainly to geography, but not to the socioeconomic status of the individual. Iodine is obtained from external supplementary sources through the consumption of food. Therefore, countries, like Jordan, could have issues concerning iodine deficiency in substantial proportions of its population, as iodine natural sources are not widely dispersed or easy to obtain. Steps

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toward providing adequate exogenous iodine supplements to decrease the prevalence of hypothyroidism, such as encouraging the use of iodide household salt, have to be followed for many decades in many countries, including Jordan (Abebe *et al*., 2017). Unfortunately, it was estimated that approximately two billion people in 2013 were either having or facing the risk of developing iodine deficiency around the world (WHO, 2014).

Since iodine is an exogenous substance, many factors influence its serum levels, including the use of non-iodide table salts, the use of unpacked salts, storing salts for lengthy periods, salt exposure to humidity and heat or sunlight, consuming foods containing goitrogens, and the presence of other nutritional deficiencies, such as iron and vitamin A deficiency can all cause low-serum concentration of iodine (Jaruratanasirikul *et al*., 2009; Kazungu *et al*., 2015; Vanderpas, 2006). These factors would precipitate low-serum levels of iodine especially affecting pregnant women and their children (Kedir *et al*., 2014). In addition, low maternal and paternal knowledge, poor economic status, history of poor maternal iodine consumption, place of residence, and large-size families are significantly associated with iodine deficiency-related conditions (Alvarez-Pedrerol *et al*., 2010).

Women, who have an iodine deficiency-related condition, may transfer this condition to their babies causing serious developmental health conditions for them. In Jordan, there is a scarcity of studies investigating this topic, leaving a gap of knowledge. Such needed studies might inform decision-makers in the country about the iodine status among this vulnerable group of the population. One relevant study was conducted by Alkafajei *et al*. (2012) in Jordan. It investigated the prevalence and risk factors of hypothyroidism among women, not specifically pregnant women, concluding that iodine deficiency is observed among a large proportion of the women in the country. Alkafajei *et al*. (2012) concluded that young aged and first-trimester women are at a higher risk for hypothyroidism as a result of iodine deficiency.

Because Jordan has limited seaside land, the iodine content of food depends mainly on the soil iodine coming from the volatile form carried into the atmosphere and returning to earth via rain, and on the dairy products (Brent, 2010). However, this source is highly variable, very slow, and does not usually deposit a significant amount of soil-based iodine (Zimmermann, 2010). Therefore, low iodine concentrations in the soil and water may result in deficiency despite the supplementation via fortified milks and iodide table salts. The International Council for the Control of Iodine Deficiency Disorders (ICCIDD) under the United Nations Children's Fund (UNICEF) recommend that the daily intake for adults must be 150 μg/day, and that this amount has to be increased to 250 μg/day for pregnant women. The European Food Safety Authority (2014) suggested a 200 μg/day daily intake as a new reference value of the adequate intake for pregnant women to avoid hypothyroidism in the mother and her fetus, and to increase the maternal renal clearance during pregnancy. Bearing in mind the need for more iodine, pregnant women in Jordan, who usually have a limited consumption of iodine, might be particularly exposed to the development of iodine deficiency which makes it necessary to have a close follow-up to diagnose and manage this health issue.

Therefore, this study was conducted to measure the level of knowledge of pregnant women concerning iodine-supported salt and iodine-rich diet during pregnancy. It also investigated the impact of this knowledge on certain dietary habits and the effect of iodine deficiency on several maternal and child complications.

2. Material and Methods

This descriptive cross-section study addressed a convenience sample of 500 Jordanian pregnant women from the north and the middle regions of the country. Participants were recruited from six governmental hospitals and two specialized maternal health clinics in six different cities in Jordan, namely Amman, Salt, Zarqa, Irbid, and Madaba, and the Northern Jordan Valley. There were efforts to recruit participants from the southern regions of the country, such as Karak, Tafileh, and Aqaba, but there were limitations.

Data were collected between November of 2014 and December of 2015. These data included demographic and previous obstetric and gynecological history-related complications of iodine deficiency, blood samples for serum TSH, FT4, and hemoglobin levels, and biophysical measurements (i.e. height and weight). These data were collected using focused interviews.

The knowledge part of the questionnaire addressed iodine dietary habits, including using iodide salts (amounts and timing), consumption of fish meals per week and knowledge concerning the impact of dietary habits on iodine serum levels and thyroid function. In addition, the questionnaire asked the participants whether they are familiar with sources of iodine, Ca interactions and iodine-deficiency-related health conditions.

According to ROCHE (Heil and Ehrhardt, 2008) reference range for free tetra-iodothyronin (FT4) in pregnancy, the first trimester FT4 range is 12.1-19.6 pmol/L, the second trimester is 9.6-17 pmol/L, and the third trimester FT4 8.4 pmol/L. Any value falling above or below these ranges is considered abnormal. All biochemical tests were completed using the ECL technique with commercially available kits (ROCHE Diagnostics, Germany).

Thyroid dysfunctions related to iodine deficiency have been defined according to the American Thyroid Association (Alexander *et al*., 2017) as follows:

Isolated hypothyroxinemia: normal maternal thyroid stimulating hormone (TSH) concentration with low FT4 in the lower 5^{th} or 10^{th} percentile of the pregnancy reference range.

*Overt hypothyroidism (OH)***:** elevated TSH (>2.5mIU) with decreased FT4 according to pregnancy reference range. Women with TSH of 10.0 mlU/L or more, irrespective of their FT4 has been considered OH.

Sub-clinical hypothyroidism (SCH): elevated TSH are based on the trimester. They are 2.51^{st} trimester, 3 in 2^{nd}, 3^{rd} trimester up to 10 mIU/L with normal FT4.

Additionally, based on the Cobland model for pregnancy risk factor assessment at Jordanian MCH centers, anemia in pregnancy is defined as the hemoglobin value being less than 11.0 g/dL (PRAMs, 1987). It is

worthwhile mentioning that there are no Jordanian, or Middle Eastern references for the FT4 and the TSH serum levels.

2.1. Statistical Analysis

For statistics, all data were computed and analyzed using SPSS version 21.0 (SPSS@ IBM). Descriptive statistics were used to describe the characteristics of the study sample and findings from the study questionnaire in terms of the numbers of health conditions related to knowledge about sources of iodine, iodine-deficiency related health conditions, and dietary habits related to the weekly intake of iodine. Normality tests were conducted on the study findings and confirmed their suitability for parametric statistical tests. Inferential statistics (i.e. t-test and ANOVA) were then used to measure the impact of dietary habits on selected maternal and child health conditions, the relationship between thyroid function on serum hemoglobin levels, and the effect of dietary habits on thyroid function.

2.2. Ethical Considerations

Permission for data collection was obtained from the Jordanian Research Ethics Committee at the Ministry of Health in Jordan, and from each center separately. The researchers explained to the candidate participants that participation in the study was voluntary. They were assured that their names would not appear anywhere in this study, and that the collected data were confidential, and would be used only by the researchers for scientific purposes. A cover letter explaining the purpose, significance of the study, and the instructions of responding to the questionnaire was attached to the study questionnaire. The researchers explained all the questions asked to the participants, who were also ensured that they can withdraw from the study anytime they feel necessary. Blood samples were collected by the researchers, who are trained and licensed registered nurses. Participants were ensured that they could get a copy of the results once prepared by the laboratory. All participants consented to participate and accepted to have a blood sample withdrawn. All participants received the results of their blood samples and the cases that required treatment were referred for further medical follow-up and management.

3. Results

3.1. Participants' Characteristics

Of the 500 participant women, the majority were less than forty years of age (Table 1). Nearly half of them were in their third trimester, and all of them had at least one previous birth. Interestingly, the proportion of women, who had previous abortions, were significantly high (45.2 %, n=226). Obese women also represented approximately half of the participants with a morbid obesity percentage of 7.4 % (n=37). It should be noted that 45 % (n=225) of the women participating in this study were in their third trimester. BMI was measured based on their actual weight after it has been calculated before pregnancy. Overweight women represented almost half the sample.

Table 1. Characteristics of the participants (n=500).

Factor	N (%)
Age	
<21	48 (9.6%)
21-30	255 (51%)
31-40	176 (35.2%)
41-47	11 (2.2%)
Trimester	
First	178 (35.6%)
Second	97 (19.4%)
Third	225 (45.0%)
Number of previous pregnancies	
1-3	224 (44.8%)
4-6	192 (38.4%)
More than 6	84 (16.8%)
Number of abortions	
0	274 (54.8%)
1-4	215 (43%)
5-8	11(2.2%)
Number of deliveries	
0	113 (22.6%)
1-4	339 (67.8%)
More than 4	48 (9.6%)
Body Mass Index	
Less than 18.9	9 (1.8%)
19-24.9	239 (47.8%)
25-29.9	150 (30%)
30-34.9	65 (13%)
More than 35	37 (7.4%)

3.2. Conditions Related to Iodine Deficiency

As illustrated in table 2, the prevalence of health conditions that are related to iodine and thyroid hypofunction during pregnancy is relatively high. Fetal deaths, for instance, have been reported to occur among 22 % (n=110) of the participants, and the number of low birth weight in babies represented 12.4 % (n=62). To ensure that these conditions are correctly reported in this study, these data were obtained from the medical records of each woman present at the hospitals after obtaining permission from the Ethics Committee at the Ministry of Health.

Table 2. Complications related to iodine deficiency.

The condition	N (%)
Number of fetal death in previous pregnancies	110 (22%)
Pre-eclampsia	45 (9%)
Placenta previa	50 (10%)
Fertility conditions	53 (10.6%)
Abruptio placenta	30 (6%)
Low weight birth	62 (12.4%)
History of fetal distress	54 (10.8%)
History of early deliveries	56 (11.2%)
Post-partum hemorrhage	39 (7.8%)
Number of children with cerebral palsy	22 (4.4%)
Number of children with learning difficulty	27 (5.4%)

3.3. Serum Thyroid and Hemoglobin Function

The majority of the participants had normal FT4 serum levels for the first and second trimesters (n=139, 78.1 % and n= 86, 88.7 %, respectively), and only 20.2 % (n=36)

of women at their first trimester had lower than normal levels (Table 3). Women in the third trimester had significantly higher than normal serum levels of FT4, but with no clinically significant symptoms appearing on them. The TSH serum levels for most of the women in their first trimester were lower or higher than the normal values. Opposite to this finding, women in their second and third trimesters had normal values, but with significantly increased ratio of high serum levels of TSH (26.8 % (n=26) and 19.6 % (n=44), respectively). Surprisingly, more than 40 % (n=206) of the women in this study were anemic (Hg < 11 gm/dl).

Table 3. Findings of the thyroid function and hemoglobin tests.

	Trimester	Level	N (%)
FT4	First	Low	36 (20.2 %)
		Normal	139 (78.1 %)
		High	3 (1.7 %)
	Second	Low	9 (9.3 %)
		Normal	86 (88.7 %)
		High	2 (2.1 %)
	Third	Lower than 8.4	18 (8.0 %)
		Higher than 8.4	207 (92.0 %)
TSH	First	Low	171 (98.3 %)
		High	3 (1.7 %)
	Second	Normal	71 (73.2 %)
		High	26 (26.8 %)
	Third	Low	7 (3.1 %)
		Normal	174 (77.3)
		High	44 (19.6 %)
Hemoglobin	Less than 11gm/dl (anemic)		206 (41.2 %)
	More than 11 gm/dl		294 (48.8 %)

3.4. Effect of Knowledge and Dietary Habits and Complications of Mother and Child

3.4.1. Fish meal per Week and Maternal or Child Conditions

Consuming weekly fish-containing meals had a statistically-significant impact on decreasing the number of fetal deaths from previous pregnancies, decreasing infertility conditions, decreasing the numbers of early deliveries, and the numbers of children with learning difficulties (Table 4).

Table 4. The Impact of weekly fish-containing meals on maternal and child health conditions.

	Mean Square	F	Sig.
Number of fetal death in previous pregnancies	1.786	10.589	**.001**
Pre-eclampsia	.003	.041	.840
Placenta previa	.177	1.962	.162
(In)Fertility conditions	.928	9.946	**.002**
Abruptio placenta	.183	3.259	.072
Low birth weight	.274	3.452	.064
History of early delivery	.417	4.213	**.041**
Post-partum hemorrhage	.050	.694	.405
Number of children with cerebral palsy	.043	1.020	.313
Number of children with learning difficulty	.298	5.869	**.016**

3.4.2. Iodide Salts and Maternal or Child Conditions

Post-partum hemorrhage and the number of children with learning difficulties decreased significantly among the women reported using iodide salts (Table 5). However, the use of iodide salts did not have any statistically significant impact on the other maternal and child health conditions investigated in this study ($P > .05$).

Table 5. The impact of using iodide salts on selected women and child health conditions.

	Mean Square	F	Sig.
No. of fetal death in previous pregnancies	.389	2.321	.128
Pre-eclampsia	.004	.045	.816
Placenta previa	.075	.834	.362
Fertility conditions	.178	1.883	.171
Abruptio placenta	.001	.011	.917
Low birth weight	.241	2.509	.114
History of early deliveries	.113	1.129	.288
Post-partum hemorrhage	.295	4.119	**.043**
Number of children with cerebral palsy	.241	2.509	.236
Number of children with learning difficulties	.346	6.847	**.009**

There was no statistically significant correlation between the weekly consumption of fish- containing meal and complications related to iodine deficiency (P=.832), TSH level (P=.415), and FT4 level (P=.517). In addition, the results showed that dietary habits (iodide salts, rich iodine diet, fish), goiter and thyroid function test did not have any statistically-significant impact on the prevalence of iodine deficiency. This finding might be related to the inadequate iodine component within these dietary materials. As illustrated in table 6, there were statistically-significant effects of FT4 and TSH serum levels on hemoglobin serum levels among pregnant women, but the results showed that higher FT4 levels were associated with higher serum hemoglobin levels.

Table 6. The impact of FT4 serum level on hemoglobin in pregnant women.

	Sum of Squares	Mean Square	F	Sig.
Free T4 * Hemoglobin	860.682	3.571	4.139	.000
TSH * Hemoglobin	690.755	2.890	1.914	.000

3.4.3. Knowledge about Iodine Role in Pregnancy and TFT

As the results show, the knowledge level among the participant women did not have any statistically-significant impact on the thyroid function tests in this study (Table 7).

Table 7. The impact of dietary habits on FT4 and TSH levels.

		Sum of Squares	Mean Square	F	Sig.
Free T4	Between Groups	370.041	370.041	.098	.755
TSH	Between Groups	72.457	72.457	.142	.706

3.5. Knowledge about the Importance of Iodine Consumption during Pregnancy

Most women (90.6 %, n=453) had very low knowledge level about the importance of iodine during pregnancy, and its relevance to several health conditions for the mothers and their babies. For the remaining small portion of the sample (9.4%, n=47), the sources of information included nursing students, the internet and mass media, books and magazines, and friends and family, respectively (Table 8). Only 27.6 % of the participating women knew about the importance of fish-containing diets as a source of iodine essential for the body mechanisms. This percentage went higher to 70.4 % when women were asked whether they were using iodide salts, but more than half of the women added these salts routinely after cooking.

Table 8. Sources of Information about Iodine, Time of Using Salts and Ca Interaction.

Item	N (%)
Source of data	
No source	453 (90.6 %)
Nursing students	25 (5.0 %)
Internet and mass media	14 (2.8 %)
Books and magazine	5 (1.0 %)
Friends, family	3 (.6 %)
Time of using salt	
Before cooking	441 (88.2 %)
After cooking	59 (11.8 %)
Ca interaction	
Poisoning	495 (99.0 %)
Iodide interaction	5 (1 %)

Women in this study did not associate the use of iodide salts to health conditions, but rather to taste. On the contrary, some women mentioned that they used salt carefully because of its impact on arterial blood pressure as they heard in the mass media (i.e. television). Women also reported that this practice of adding iodide salts after cooking was not based on their awareness of the significance of iodine, but for taste preferences. A few number of women (10 %) knew about the complications of iodine deficiency, iodine supplements (1 %), oil fish (3.6 %), and only 1.4 % of the women knew about goitrogenics (Table 9).

Table 9. Iodine containing food consumption and knowledge about iodine-deficiency related conditions.

Item	NO	YES
Consumption of fish within the meal/week	362 (72.4%)	138 (27.6%)
Iodide salt	148 (29.6%)	352 (70.4%)
Iodine supplement	495 (99.0%)	5 (1%)
Supplement of oil fish	482 (96.4%)	18 (3.6%)
Knowledge about goitrogenic	493 (98.6%)	7 (1.4%)
Knowledge about goiter	488 (97.6%)	12 (2.4%)
Knowledge about complications related to iodine deficiency	448 (89.6%)	52 (10.4%)

4. Discussion

This study showed that the knowledge level and dietary habits of women to consume iodine and avoid iodine-related health conditions are still below the needed levels. This appears clearly from the relatively substantial proportion of women, who were reported having a history of different problems during their previous pregnancies, including fetal deaths and abortion (see table 1). Although women in this study were young, fertile, and did not have major chronic or acute health conditions, such as hypertension and diabetes mellitus, many of them reported passing through major challenges in the past without realizing that these conditions were related to iodine-deficiency and hypothyroidism. Clearly, many agencies within Europe and North America emphasize the importance of providing supplemental iodine for pregnant women as well as for children during their early school levels (EFSA, 2014; WHO, 2014). However, similar emphasis could not be seen in Jordan.

Women in this study were aged less than 47 year/old, but approximately half of them reported having major challenges in previous pregnancies, experiencing delayed pregnancy (secondary to infertility with no apparent reasons), and had conditions during or after delivery. Comparable findings, but with a relatively higher age of women, have been reported by other studies, such as Almomin *et al*. (2016), and Bocos-Terraz *et al*. (2009). Therefore, it is crucial to improve the food habits of pregnant women with regard to the iodine consumption especially that nearly all the participants in this study expressed their plan to have more babies in the future. Based on the findings of the current study, women need to receive adequate knowledge about the role of iodine in the thyroid function and body mechanisms, in addition to information about the dietary sources of iodine, the conditions associated with low-serum iodine and other issues important for the health of the mother and her fetus.

The results of this study indicated the presence of seventy-three women with TSH levels higher than normal, and sixty-three women with FT4 serum levels below the international refereed normative values for the three trimesters. However, only fifty-two women required medical follow-up, and the remaining number of women were prescribed supplements with no clinically significant symptoms. According to Alexander *et al*. (2017), referenced intervals for TSH and FT4 vary significantly in different populations. Examples of the variation could be

found in the TSH serum levels reported by different agencies in the world. In Japan, for example, it was observed that healthy pregnant women with a normal thyroid function had levels lower than .6 mU/L (Orito *et al*., 2009, while in the USA the levels were below .4 mU/L (Kahric-Janicic *et al*., 2007). The thyroid gland is overstimulated during pregnancy resulting in changes in the concentration and excretion of its hormones (Soldin, 2006). Again, the proper and adequate knowledge about the impact of iodine, its sources, and symptoms related to its deficiency is essential to those women even prior to pregnancy so as to avoid transferring this deficiency to the born children.

Findings also indicated that more than 40 % (n=206) of the women had serum hemoglobin levels below 11 mg/dl, which fell under the anemia category according to the guidelines. This finding has not been well-studied in the literature, but it could be related to several factors, including dietary habits and the expansion of the mother circulation (Bencaiova and Breymann, 2014). In part, it could also be a manifestation related to hypothyroidism and the decreased serum iodine in pregnant women (Erdogan *et al*., 2012), especially when anemia was found alongside all trimesters and did not cluster at one interval of pregnancy.

The frequent use of iodide salts (with no definite amount) has been associated positively with a decreased prevalence of post-partum hemorrhage in this study. Unfortunately, urinary iodine concentration has not been investigated to reflect the real picture clearly due to limited resources. We also found that the weekly consumption of a fish meal was positively associated with a decreased number of fetal deaths, infertility and premature deliveries. Kim *et al*. (2014) investigated iodine deficiency in the Philippines, a country high in its resources of sea food. They have found that iodine deficiency exists among school children despite the governmental efforts to eliminate this condition. Kim *et al*. (2014) argued that most students, especially the females, did not acknowledge the importance of iodine deficiency for pregnant women and their babies. The limited knowledge, as this study shows, partially led to iodine deficiency health-related conditions during pregnancy. In a country like Jordan with limited seafood resources, further efforts should be directed to improve the level of knowledge and the dietary habits to increase the awareness, and decrease the conditions associated with the iodine deficiency during the early stages of life, such as school years.

A very interesting point in this study was related to the knowledge component in relation to the iodine role in the health of the mother and her fetus (child after delivery). Although women reported that it was important to improve their knowledge about iodine, they did not report obtaining information from reliable sources. In fact, most women (90.6 %, n= 453) did not seek any information about iodine. The remaining portion of the sample (9.4 %, n=47) obtained their information mainly from students, the internet and mass media. The information received by the participants could be unreliable or even misinterpreted. Therefore, there is a need to have control over the type of information provided to pregnant women and the public by providing alternative and more reliable resources, such school books and brochures developed and distributed by the ministry of health or any other specialized health agency. Similarly, Garnweidner-Holme *et al*. (2017) reported comparable results when they assessed information about iodine during pregnancy and lactation among Norwegian women. But Garnweidner-Holme *et al*. (2017) indicated that well-educated women and those who received education on the iodine role in pregnancy scored significantly higher mean scores than those who did not. This finding indicates that the learning sessions of the females could improve the knowledge level and practice concerning the iodine consumption during pregnancy and lactation (O'Kane *et al*., 2016).

Women reported that fish was an important source of iodine, but most of them did not know that iodine deficiency is associated with maternal and child health conditions. The practice of consuming higher amounts of food rich in iodine by women could be improved when providing them with information about the [good] sources of iodine, and what serious impacts iodine could have on their babies' health supported by evidence from the literature. Such serious impacts include future child learning difficulties resulting from delayed neurological development (Hynes *et al*., 2013). Combet *et al*. (2015) reported in a study that included 1026 women in the UK that women knowledge concerning nutritional requirements was high (96 %) reflecting an adequacy of knowledge and healthy practices. However, iodine knowledge was low compared with other nutrients (12 %). In addition, Combet *et al*. reported that the median pregnancy iodine intake was 190 µg/day, which is lower than the 2014 WHO recommended intake for pregnant women of 250 µg/day. They concluded that dietary recommendations during pregnancy in the UK and their dissemination did not supply women with the needed requirements of iodine (Combet *et al*., 2015). Evidence from the literature determines the impact of education on women compliance to iodine rich diets in order to avoid health conditions associated with iodine deficiency during pregnancy and in the aftermath.

A very limited number of the women had iodine (1%, n=5) and oil fish (3.6%, n=18) supplements prior to the study. Perhaps the participants never tested iodine and the thyroid state until being enrolled in this study and discovering they had a deficiency. Similarly, the women did not appear to have adequate knowledge and healthy practices concerning their iodine intake, which might have caused a reasonably high prevalence of different complications during their previous pregnancies. Comparable conclusions were made by authors, like Hynes *et al*. (2013) and Delange (2007). A study by Charlton *et al*. (2010) addressed knowledge level and practice of pregnant women in Australia, and concluded that there was an urgent need for decision-makers to set public health strategies guiding nutrition, education and supplementation to improve the pregnant women iodine-state. The current study found that information provision is extremely limited for women during and before pregnancy. This finding indicates the need to develop readily accessible sources of information on iodine, especially for women attending maternal and child health centers.

5. Limitations

The number of women who participated in this study was relatively low and did not represent all regions of Jordan. Longitudinal studies would achieve more tentative results reflecting the development of pregnant women health over the three trimesters and during breastfeeding, which cross-sectional studies, like this one, usually misses. The use of drugs interfering with iodine serum levels has been screened by a single question asking directly about the medications that these women took regularly. This point needs further elaborative questions, which would determine the drugs women might have taken not knowing they would influence iodine and the thyroid function. In addition, the impact of overweight on iodine levels and the thyroid function was not covered in this study due to limitations in resources.

In addition, this study did not address urinary iodine levels as an indicator of thyroid function among pregnant women. It also did not explore items like fish meal consumption in more details. This study questioned women about the weekly fishmeal, but did not ask about the type fish, how it was consumed (raw, cooked, etc.), and the amount consumed by the women. This is an important question since there is a wide range of iodine levels in the diverse fish types. Furthermore, the method of storing and cooking plays a key role in maintaining the iodine composition with the fish. In addition, due to the substantial proportion of women, who had hemoglobin less than 11mg/dl, there was a need to perform anemia tests, such as serum iron and folic acid, all of which could interfere with the iodine serum levels.

6. Conclusions and Recommendations

Findings in this study indicated that the participating women were not aware of the impact of iodine on their pregnancy and their babies. Educational programs and materials should be made available for women in the clinics. Schools should also be involved in a national program to raise the students' awareness regarding the impact of iodine on the mother and her child. In addition, the role of mass media in increasing this awareness is very important as it could reach even the most remote areas in the country. Therefore, further efforts should be made to present educative materials for women utilizing all available tools of communication.

It has been widely reported in the literature that iodine deficiency is among the most prevalent diet-related health conditions in the world. The need for extra supplements of iodine for pregnant women has been emphasized by many organizations across the USA and Europe. Similar emphasis is needed in Jordan. The impact of adding iodine to table salts and other iodine-rich food products on decreasing the prevalence of hypothyroidism, particularly amongst pregnant women, is well-documented. However, comparable results have not been found in this study. In Jordan, there is a need to ensure that adequate iodine is added to the dietary preparations. Furthermore, as Jordan is limited in its resources of seafood, alternative supplementary foods should be considered for mothers and children, such as oil fish supplements. There is a need to include biomarkers in the assessment of iodine levels among pregnant women, including dietary assessments (food diaries, or 24-h food intake, recall and weighed food records), urinary iodine concentration (UIC), TSH, thyroglobulin, T4/FT4, and goiter assessment.

Finally, this study had some limitations since it did not address issues concerning the dietary habits, amounts of iodide salts used, frequency of using iodide salts, and the consumption of seafood by the pregnant women and history of thyroid conditions. Therefore, we recommend the conduction of further studies addressing TFT levels among pregnant women. The formulation of a national policy development of supplementary iodine and fish oil preparations for pregnant women.

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Molecular Typing and Detection of Collagen Binding Genes among *Streptococcus mutans* Isolated from Diabetic and Non-diabetic Individuals in Northern Jordan

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Abstract

Streptococcus mutans, a cause of dental caries, is an opportunistic pathogen leading to subacute infective endocarditis and bacteremia. Bacterial attachment to the heart valves, requires the presence of collagen-binding proteins (CBP), such as Cnm and Cbm; encoded by *cnm* and *cbm* genes, respectively. Herein, the prevalent *S. mutans* serotypes and genes for CBPs among diabetics and non-diabetic controls from Northern Jordan were investigated. Teeth swabs were cultured on TYCSB agar for *S. mutans* isolation. PCR was used to confirm the isolates' identity, and to identify the isolates' serotypes and CBP genes. The most prevalent serotype among the diabetics was c (71.6 %), followed by k (43.2 %), f (32.1 %), and e (13.6 %). Among the diabetics, 44.4 % harbored two or more serotypes. The most prevalent serotype among the non-diabetics was k (92.1 %), followed by c (69.8 %), f (25.4 %), and e (15.9 %). Among the non-diabetics, 92.1 % harbored two or more serotypes. The prevalence rates for *cnm* and *cbm* were 75.3 % and 11.1 %, respectively among the diabetics' isolates, and 38.1 % each among the non-diabetics' isolates. Due to the high prevalence of serotype k and the isolates harboring CBP genes, the Jordanian population maybe at the risk of developing *S. mutans*-related complications, such as infective endocarditis.

Keywords: *Streptococcus mutans*, Prevalence, Serotypes, Collagen-binding protein, Diabetes

1. Introduction

The mouth of healthy humans is colonized by a huge number of microorganisms, the majority of which constitute the bacterial normal flora. These microorganisms compete with other microbes for food and colonization sites, and act as a part of the host immune defense (Aas *et al.*, 2005, Forssten *et al.*, 2010). The oral bacteria include streptococci, staphylococci, lactobacilli, and various anaerobes such as *Bacteroides* spp. (Aas *et al.*, 2005).

Streptococcus mutans is a Gram-positive facultative anaerobic bacterium capable of tolerating O_2. It is capable of surviving at a temperature range of 18-40 ^oC. It is an important member of the oral normal flora in most individuals. Colonization of the mouth, and more specifically, the dental plaque begins after the eruption of teeth (Liljemark and Bloomquist, 1996). *S. mutans* is capable of metabolizing sucrose to form high molecular weight polysaccharides using the glucosyltransferase enzyme, which allows the bacterium to initiate plague formation (i.e., biofilms) on the teeth's enamel surface. The enamel is the external visible part of the teeth, and the hardest substance in the human body as it contains a very high concentration of minerals. Unless quickly removed, plaques will grow in size and become very difficult to eliminate. The plaques are composed of food debris, bacteria (mainly *S. mutans*), and extracellular products. *S. mutans* is the leading cause of dental caries worldwide because it metabolizes several carbohydrates, creating lactic acid that demineralizes teeth enamel (Usha and R, 2009, Forssten *et al.*, 2010, Esberg *et al.*, 2017). Following the destruction of enamel, the bacteria utilize digestive enzymes to degrade the protein matrix found in the dentin and cement layers of teeth (Loesche, 1986).

S. mutans is classified into four main serotypes; c, e, f, and k, based on the identity of surface rhamnose-glucose polymers (RGPs). Serotype c strains are the most common in the oral cavity (Nakano *et al.*, 2007). The RGPs are important components of the cell wall of streptococci and other bacterial species. The polymers of α1,2- and α1,3-linked rhamnose units form the backbones of the RGPs and the polysaccharide antigens of Lancefield group A, C, and E streptococci. However, little is known about the mechanisms of synthesis of these polysaccharides (Shibata *et al.*, 2002).

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S. mutans is an opportunistic pathogen capable of causing infective endocarditis and bacteremia (Nakano *et al.*, 2007). Transient but harmless, bacteremia can occur following the brushing and flossing of teeth, tooth extractions, and other dental procedures (Forner *et al.*, 2006). The presence of *S. mutans* in the blood stream allows bacterial colonization of the inner lining of the heart, the endocardium, and the heart valves, potentially leading to damaged heart valves (endocarditis) and other complications (Nomura *et al.*, 2006). Endocarditis could lead to a progressive destruction and damage of the affected heart tissue, and may lead to other complications such as vertebral infections, bacterial pneumonia, and hemorrhagic stroke (Nakano *et al.*, 2006, Biswas *et al.*, 2010, Mansour *et al.*, 2017, St Michael *et al.*, 2017). Endocarditis, if untreated, is a life- threatening condition, therefore, individuals with damaged heart valves are instructed to take antimicrobials prophylactically before undergoing dental procedures or a surgery (Nakano *et al.*, 2007).

The bacterial binding and colonization of the extracellular matrix, is the first step in the invasion of host cells. The ability of *S. mutans* to attach to surfaces requires the presence of collagen-binding proteins (CBPs). To date, two CBPs, namely Cnm and Cbm, have been identified, with Cbm typically being more common than Cnm (Nomura *et al.*, 2013). Parts of the extracellular matrix that are most commonly colonized by bacteria are collagen, fibronectin, and laminin. The 120KD Cnm protein possesses binding activity to type I collagen and laminin (Nomura *et al.*, 2009, Abranches *et al.*, 2011). CBPs not only allow the efficient colonization of dental pulp, but possibly other tissues as well (Nomura *et al.*, 2016).

The CBPs are not only involved in teeth decay, but are now recognized as important virulence factors allowing the virulent strains to invade tissue, be attached to surfaces, invade endothelial cells, and cause infective endocarditis. In support for the increased virulence of strains expressing CBPs, it was found that CBP-expressing strains, such as most serotype k isolates ,were accumulated in damaged blood vessels and resulted in increased tissue damage when tested in a mouse cerebral hemorrhage model (Nakano *et al.*, 2007, Abranches *et al.*, 2011, Nakano *et al.*, 2011, Palmer *et al.*, 2013).

Diabetic patients have higher blood and saliva sugar levels than non-diabetics, which may potentially affect the constituents of the bacterial oral flora (Vernillo, 2003). Hence, the researchers hypothesized the presence of *S. mutans* serotype and genotype differences among diabetics and non-diabetic controls. Several studies have investigated the potential differences in *S. mutans* carriage among diabetics and non-diabetics (Hintao *et al.*, 2007, Nomura *et al.*, 2009, De *et al.*, 2016, Rezazadeh *et al.*, 2016). In this study the most frequent *S. mutans* serotypes among diabetic and non-diabetic individuals from Northern Jordan, and the molecular prevalence of *S. mutans* strains possessing collagen binding protein genes have been determined.

2. Materials and Methods

2.1. Sample Collection and Bacterial Isolation

The current study was approved by the Institutional Review Board of Jordan University of Science and Technology. One hundred diabetics (type 2), and seventy-seven non-diabetics, from both genders and all age groups were recruited for this study using judgmental sampling method. Informed written consents from the participants or their legal guardians, were obtained before enrollment in this study. A teeth surface material was obtained from each participant by swabbing the teeth surfaces using a sterile cotton swab. The swabs were passed on the teeth surfaces and buccal areas of molars to obtain the bacterial samples. The swabs were immediately cultured on tryptone-yeast-cysteine agar medium (Lab M, UK) supplemented with 20 % sucrose and 0.2 U/mL bacitracin (TYCSB agar); i.e., primary culture. The TYCSB agar medium is selective for *S. mutans* and provides excellent recovery of *S. mutans* from teeth swabs (Wan *et al.*, 2002). All the secondary subcultures of *S. mutans* were done utilizing Mueller Hinton agar media (Scharlan Microbiology, Spain). Since many *S. mutans* serotypes could colonize the same individual, several *S. mutans* colonies from each primary culture were processed during all cultural manipulations to identify all possible serotypes and CBP genes using the PCR protocols below. All primary and secondary cultures were incubated aerobically at 37 °C with 5% CO_2 for 48 h.

2.2. Streptococcus mutans Identification

Preliminary isolates identification following growth on TYCSB selective media was based on obtaining a negative catalase test and observing characteristic bacterial morphology and Gram-reaction. Definitive isolate identification was done using a commercially available kit (liofilchem® Strepto-System 9R, Italy) according to manufacturer instructions.

2.3. DNA Extraction

Bacterial suspensions were prepared for total (chromosomal and plasmid) DNA extraction of *S. mutans* isolates. Briefly, several pure bacterial colonies were inoculated into 3 mL of brain heart infusion (BHI) broth, and tubes were incubated aerobically overnight at 37 ºC in the presence of 5 % CO_2.

Genomic DNA extraction was performed from 1 mL overnight BHI cultures using E.Z.N.A® Bacterial DNA kit (OMEGA bio-tek, USA), in accordance with the manufacturer instructions. DNA was eluted using 50 µL of elution buffer. DNA was stored at -20 ºC for later use in PCR.

2.4. PCR Assays

Genomic DNA from all the isolates was subjected to multiple PCR assays to confirm the identity of isolates (using species specific genes; *gtfD* and *gtfB*), determine isolates' serotypes, and to identify the presence of CBP genes (*cbm* and *cnm*). Table 1 indicates the primers used for PCR.

Table 1. Primers used for PCR amplification.

Primer	Purpose	Sequence (5`-3`)	Amplicon size (bp)	Reference
gtfD-F	*S. mutans* detection	GGCACCACAACATTGGGAAGCTCAGTT	433	(Hoshino *et al.*, 2004)
gtfD-R		GGAATGCCGATCAGTCAACAGGAT http://jmm.sgmjournals.org/content/58/4/469/T1.expansion.html - ref-10		
gtfB-F	*S. mutans* detection	ACTACACTTTCGGGTGGCTTGG	517	(Oho *et al.*, 2000)
gtfB-R		CAGTATAAGCGCCAGTTTCATC		
SC-F	Serotype c detection	CGGAGTGCTTTTTACAAGTGCTGG	727	(Shibata *et al.*, 2003)
SC-R		AACCACGGCCAGCAAACCCTTTAT		
SE-F	Serotype e detection	CCTGCTTTTCAAGTACCTTTCGCC	517	(Shibata *et al.*, 2003)
SE-R		CTGCTTGCCAAGCCCTACTAGAAA		
SF-F	Serotype f detection	CCCACAATTGGCTTCAAGAGGAGA	316	(Shibata *et al.*, 2003)
SF-R		TGCGAAACCATAAGCATAGCGAGG		
CEFK-F	Serotype k detection	ATTCCCGCCGTTGGACCATTCC	294	(Nakano *et al.*, 2004)
K-R		CCAATGTGATTCATCCCATACC		
cbm-EF	*Cbm* detection	AGCTGAAGTTAGTGTTGTAAAACCTGCTTC	393	(Nomura *et al.*, 2012)
cbm-ER		TAGGATCATCAACCTTAGTCAAGTACACGA		
cnm-DF	*Cnm* detection	TGGAGGTTCAGGGCAAGTATGTTGGTGATT	579	(Nomura *et al.*, 2012)
cnm-DR		GTCTTTTGATCAGGATTGTCAACTTTAGTC		

2.4.1. PCR for Streptococcus mutans Identity Confirmation and Serotype k Detection

PCR confirmation of the *S. mutans* isolate identity was done using the species-specific *gtfB* and *gtfD* gene primers. For *gtfB* PCR, amplification conditions consisted of 95 ºC for four minutes; thirty cycles of 95 ºC for thirty seconds, 59 ºC for thirty seconds, and 72 ºC for one minute, and 72 ºC for seven minutes. For *gtfD* PCR, amplification conditions consisted of 98 ºC for four minutes; thirty cycles of 98 ºC for ten seconds and 70 ºC for one minute, and 70 ºC for seven minutes. For each of the two assays, each 25 µL PCR tube contained 12.5 µL of 2x PCR master mix solution (i-MAX II, iNtRON Biotechnology, South Korea), 3 µL of template DNA, 0.75 µL of each primer (10 pmoles/µL), and 8 µL of nuclease-free water. *S. mutans* ATCC 25175 was used as positive control. The negative control consisted of a reaction without the addition of template DNA.

PCR identification of *S. mutans* serotype k was done using the primer pair CEFK-F/K-R. Amplification conditions consisted of 95 ºC for four minutes, thirty cycles of 95 ºC for thirty seconds, 60 ºC for thirty seconds, and 72 ºC for thirty seconds, and 72 ºC for seven minutes. Each 25 µL PCR tube contained 12.5 µL of 2x PCR master mix solution (i-MAX II), 2 µL of template DNA, 0.75 µL of each primer (10 pmoles/µL), and 9 µL of nuclease-free water. The negative control consisted of a reaction without the addition of template DNA.

2.4.2. Multiplex PCR for Detection of Streptococcus mutans Serotypes c, e, and f

Multiplex PCR for the detection of *S. mutans* serotypes c, e, and f utilized the respective primers indicated in table 1. PCR consisted of 96 ºC for two minutes, twent-five cycles of 96 ºC for fifteen seconds, 61 ºC for thirty seconds, and one minute at 72 ºC, and 72 ºC for five minutes. Each 25 µL PCR tube contained 12.5 µL of 2x PCR master mix solution (i-MAX II), 3 µL of template DNA solution, 1.25 of each of the respective primers (10 pmoles/µL), and 2 µL of nuclease-free water. *S. mutans* ATCC 25175 was used as positive control. The negative control consisted of a reaction without the addition of template DNA.

2.4.3. Multiplex PCR for the Detection of Streptococcus mutans Collagen Binding Protein Genes

Multiplex PCR for the detection *S. mutans* CBP genes; *cbm* and *cnm* utilized the primers indicated in table 1. PCR consisted of 95 ºC for four minutes, thirty cycles of 94 ºC for thirty seconds, 60 ºC for thirty seconds, and 72 ºC for thirty seconds, and 72 ºC for seven minutes. Each 25 µL PCR tube contained 12.5 µL of 2x PCR master mix solution (i-MAX II), 3 µL of template DNA, 0.75 µL of each primer (10 pmoles/µL), and 6.5 µL of nuclease-free water.

2.4.4. Agarose Gel Electrophoresis

PCR amplification products were identified following electrophoretic separation on 2 % agarose gels containing ethidium bromide at 140 volts for forty minutes. Five microliters of each PCR product was used per gel lane. DNA bands were visualized using a UV transilluminator provided with a gel documentation system using the Quantity One software (Biorad, USA). Fragment sizes for each PCR product were determined by comparison with a 100 bp DNA ladder (Quick-load DNA ladder, Cat #N0467S, New England Biolabs, USA) and with the positive control when available.

2.5. Statistics

The Statistical Package for Social Sciences (SPSS) software (version 23) (IBM, USA) was used for the statistical analysis of data. Frequency results were compared using the Chi-Square test. A *P* value less than 0.05 was considered statistically significant.

3. Results

3.1. Identification of Streptococcus mutans

Eighty-one out of one-hundred samples among the diabetics, as well as sixty-three out of seventy-seven of the non-diabetics' samples, were positive for *S. mutans*. Identification was based on morphologic, phenotypic, biochemical characteristics, and the detection of the *S. mutans* specific genes *gtfB* and/or *gtfD*.

3.2. Study Participants

The eighty-one diabetics having *S. mutans* had a mean age of 52.9 years with a standard deviation of 11.7 years, and a range of twelve to sixty-nine years. The sixty-three non-diabetics having *S. mutans* had a mean age of thirty-four years with a standard deviation of 18.7 years, and a range of five to seventy-two years. Table 2 demonstrates the distribution of participants according to gender and arbitrary age groups.

Table 2. Gender and age group distribution of the study groups.

	Age	Age group (Years)		
Criteria	Mean (Years)	< 30 Count (%)*	30 - 59 Count (%)*	> 50 Count (%)*
Diabetics	Female 53	2 (2.5%)	29 (35.8%)	16 (19.7%)
	Male 52	1 (1.2%)	22 (27.2%)	11 (13.6%)
Non-diabetics	Female 32	15 (23.8%)	14 (22.2%)	5 (7.9%)
	Male 36	13 (20.6%)	11 (17.4%)	5 (7.9%)

* Percentages were calculated out of the total number among each study group.

3.3. Streptococcus mutans Serotypes and Collage Binding Protein Genes

Among the diabetics group, serotype c was the most frequently encountered (71.6 %), followed by k at 43.2 %, f at 32.1 %, and e at 13.6 %. No significant distribution differences were observed in the three indicated age groups (Table 3). *Cnm* had the highest prevalence rate at 75.3 %, followed by the *cbm* at 11.1 % (Table 3).

Among the non-diabetics group, serotype k was the most frequently encountered (92.1 %), followed by c at 69.8 %, f at 25.4 %, and serotype e at 15.9 %. A statistically significant difference (P = 0.028) in the distribution of serotype c was observed among the three age groups with prevalence occurring at a higher rate in the young and the old age groups compared to the middle age group (Table 4). *Cnm* and *cbm* were identified at the same rate (38.1 %) (Table 4).

A statistically significant difference in the distribution of serotype k, *cnm*, and *cbm* was observed among the two study groups. Serotype k and *cbm* were more prevalent among the non-diabetics group; 92.1 % vs. 43.2 %, and 38.1 % vs. 11.1 %, respectively. In contrast, *cnm* was more prevalent among the diabetics group; 75.3 % vs. 38.1 % (Table 5). Among the diabetics group isolates, *cnm* and *cbm* were most prevalent with serotype c. Among non-diabetics group isolates, *cnm* and *cbm* were most prevalent with serotypes k and c (Table 6). However, no statistically significant associations were observed between each of *cnm* and *cbm*, and each of the different serotypes, among each of the study groups (Table 6).

Table 3. Distribution of serotypes and collagen binding protein genes according to age among the diabetics group.

Criteria		Age group (Years) < 30 Count (%)*	30 - 59 Count (%)*	> 59 Count (%)*	*P* value	Total (%)
Serotype k	Absent	2	29	15	0.934	46
	Present (%)	1 (33.3%)	22 (43.1%)	12 (44.4%)		35 (43.2%)
Serotype c	Absent	1	14	8	0.961	23
	Present (%)	2 (66.7%)	37 (72.5%)	19 (70.4%)		58 (71.6%)
Serotype e	Absent	3	45	22	0.556	70
	Present (%)	0 (0%)	6 (11.8%)	5 (18.5%)		11 (13.6%)
Serotype f	Absent	2	37	16	0.489	55
	Present (%)	1 (33.3%)	14 (27.5%)	11 (40.7%)		26 (32.1%)
Serotype	c (%)	1	19	11	0.926	31 (49.2%)
	c + e + f (%)	0	1	0		1 (1.2%)
	C + e + f + k (%)	0	1	3		4 (4.9%)
	c + f (%)	0	1	0		1 (1.2%)
	c + f + k (%)	0	3	1		4 (4.9%)
	c + k (%)	1	12	4		17 (21%)
	e (%)	0	2	1		3 (3.7%)
	e + f (%)	0	1	1		2 (2.5%)
	e + k (%)	0	1	0		1 (1.2%)
	f (%)	1	5	2		8 (9.9%)
	f + k (%)	0	2	4		6 (7.4%)
	k (%)	0	3	0		3 (3.7%)
Cnm	Absent	1	14	5	0.643	
	Present (%)	2 (66.7%)	37 (72.5%)	22 (81.5%)		61 (75.3%)
Cbm	Absent	3	45	24	0.820	
	Present (%)	0	6 (11.7%)	3 (11.1%)		9 (11.1%)

* Percentages were calculated out of the total number for each age group.

Table 4. Distribution of serotypes and collagen binding protein genes according to age among the non-diabetics group.

Criteria		Age group (Years) < 30 Count (%)*	30 - 59 Count (%)*	> 59 Count (%)*	*P* value	Total (%)
Serotype k	Absent	2	2	1	0.960	5
	Present (%)	26 (92.9%)	23 (92%)	9 (90%)		58 (92.1%)
Serotype c	Absent	4	12	3	0.028	19
	Present (%)	24 (85.7%)	13 (52%)	7 (70%)		44 (69.8%)
Serotype e	Absent	25	18	10	0.074	53
	Present (%)	3 (10.7%)	7 (28%)	0		10 (15.9%)
Serotype f	Absent	23	17	7	0.466	47
	Present (%)	5 (17.9%)	8 (32%)	3 (3%)		16 (25.4%)
Serotypes	c (%)	1	1	0	0.353	2(3.2%)
	c + e + f + k (%)	1	1	0		2 (3.2%)
	c + f + k (%)	0	1	0		1 (1.6%)
	c + f + k (%)	2	0	0		2 (3.2%)
	c + k (%)	20	10	7		37 (58.7%)
	e + k (%)	2	5	0		7 (11.1%)
	f (%)	1	1	1		3 (4.8%)
	f + k (%)	1	6	2		9 (14.3%)
Cnm	Absent	16	14	9	0.136	39
	Present (%)	12 (42.9%)	11 (44%)	1 (10%)		24 (38.1%)
Cbm	Absent	14	16	9	0.079	39
	Present (%)	14 (50%)	9 (36%)	1 (10%)		24 (38.1%)

* Percentages were calculated out of the total number for each age group.

Table 5. Distribution of serotypes and collagen binding protein genes among study groups' isolates.

Criteria		Group Diabetics Count (%)*	Non-diabetics Count (%)*	*P* value
Serotype k	Absent	0	5	0.000
	Present (%)	35 (43.2%)	58 (92.1%)	
Serotype c	Absent	23	19	0.817
	Present (%)	58 (71.6%)	44 (69.8%)	
Serotype e	Absent	70	53	0.699
	Present (%)	11 (13.6%)	10 (15.9%)	
Serotype f	Absent	55	47	0.380
	Present (%)	26 (32.1%)	16 (25.4%)	
Cnm	Absent	20	39	0.000
	Present (%)	61 (75.3%)	24 (38.1%)	
Cbm	Absent	72	39	0.000
	Present (%)	9 (11.1%)	24 (38.1%)	

Table 6. Distribution of collagen binding protein genes among *S. mutans* serotypes of the study groups.

Criteria			*cnm* Absent Count	Present Count (%)*	*P* value	*cbm* Absent Count	Present Count (%)*	*P* value
Diabetics	Serotype k	Absent	11	35	0.852	39	7	0.178
		Present (%)	9	26 (42.6%)		33	2 (22.2%)	
	Serotype c	Absent	6	17	0.854	21	2	0.663
		Present (%)	14	44 (72.1%)		51	7 (77.7%)	
	Serotype e	Absent	19	51	0.197	62	8	0.819
		Present (%)	1	10 (16.4%)		10	1 (11.1%)	
	Serotype f	Absent	17	38	0.059	48	7	0.501
		Present (%)	3	23 (37.7%)		24	2 (22.2%)	
Non-diabetics	Serotype k	Absent	5	0	0.068	4	1	0.385
		Present (%)	34	24 (100%)		35	23 (95.8%)	
	Serotype c	Absent	12	7	0.893	11	8	0.667
		Present (%)	27	17 (70.8%)		28	16 (66.7%)	
	Serotype e	Absent	35	18	0.120	34	19	0.398
		Present (%)	4	6 (25%)		5	5 (20.8%)	
	Serotype f	Absent	29	18	0.955	29	18	0.955
		Present (%)	10	6 (25%)		10	6 (25%)	

***** The percentages were calculated out of the total number for each collagen binding protein gene.

4. Discussion

Oral health is one of the determinants of the quality of life. It limits and prevents oral diseases that arise from different microbial species colonizing the oral cavity. These organisms tend to form dental plaque biofilms. *S. mutans* is the most frequent species in dental plaques. It has several common serotypes, namely c, e, f, and k. Some isolates also carry genes for CBPs, which enable the bacterial binding and colonization of the heart endocardium and damaged heart valves, leading to infective endocarditis and bacteremia. In this study, the prevalent serotypes and the major virulence CBP genes of *S. mutans* isolated from the teeth of diabetic and non-diabetic subjects from Northern Jordan were investigated.

Teeth of diabetics and non-diabetics were swabbed, and the swabs were used to inoculate TYCSB medium, one of the best media for isolation of *S. mutans* (Wan *et al.*, 2002). After growth and identification, multiple bacterial colonies were taken from each plate to assure the recovery of multiple serotypes, if present. Bacterial DNA was extracted and used in several PCR assays using primers specific for *S. mutans*, its serotypes, and CBP genes.

Among the diabetics group, 81 % (81/100) of the subjects sampled were positive for *S. mutans*. All isolates were positive for at-least one of the species-specific *S. mutans* genes (*gtfB* and *gtfD*). The most prevalent serotype among this group was serotype c (71.6 %), followed by k (43.2 %), f (32.1 %), and e (13.6 %). Many subjects harbored several *S. mutans* serotypes, namely, thirty-six subjects (44.4 %) had two or more serotypes. No significant serotype distribution differences were observed in the three indicated age groups. A recent study from Italy reported serotype c as the major serotype among diabetics at 82 %, which is consistent with the findings of the current study, followed by e at 11.8 %, and f at 5.9 % (De *et al.*, 2016). However, the study from Italy utilized a small sample size (seventeen total isolates). Hence, the reported results may not be highly representative.

Among the non-diabetics group, 81.8 % (63/77) of the subjects sampled were positive for *S. mutans*. All isolates were positive for at-least one of the species-specific *S. mutans* genes (*gtfB* and *gtfD*). The most common serotype among the participants was serotype k (92.1 %; 58/63), followed by c (69.8 %), f (25.4 %; 16/63), and e (15.9 %; 10/63). Most subjects harbored several *S. mutans* serotypes; namely, fifty-eight subjects (92.1 %) had two or more serotypes. A statistically significant difference (P = 0.028) in the distribution of serotype c was observed among the three age groups with prevalence occurring at a higher rate in the young and the old age groups compared to the middle age group. Such differences may be related to dietary differences or differences in oral-hygiene practices according to age.

Studies from Japan, Europe, and North America, which investigated the prevalence of *S. mutans* serotypes among healthy individuals, reported that serotype c was the most-frequent, occurring at a prevalence rate of 70-80 % (Nomura *et al.*, 2009). Similarly, serotype c was also present in the current study at rates similar to those reported worldwide (i.e., 71.6 % and 69.8 % among the diabetics and non-diabetics groups, respectively) (Nomura *et al.*, 2009). In contrast to other reports, serotype k was the most frequently identified serotype among the non-diabetics group (92.1 %), and the second most frequent among the diabetics at 43.2 %. The difference in serotype k prevalence among the two groups was statistically significant. Differences observed regarding serotype k prevalence from that of the previous reports could be attributed to population, socioeconomic, and life-style differences, use of different sampling sites, methods of cultivation and isolation, differences in sugars intake and eating habits, and differences in oral hygiene practices. Furthermore, serotype k was more prevalent among the controls than the diabetics. This difference could be attributed to the higher blood sugar levels (and consequently higher saliva sugar levels), and other clinical and physiological differences among the diabetics, compared to the non-diabetics.

A strong relationship has been reported between serotype k and infective endocarditis. This may be attributed to a reduction in the content of glucose side chains in its RGPs, leading to less solubility in blood and higher resistance to phagocytosis by neutrophils (Nakano *et al.*, 2004). Studies from Japan and the UK, reported the entry of serotype k *S. mutans* to the circulation following dental procedures, when no antimicrobial agents were administered for prophylaxis (Nakano *et al.*, 2004, Nakano *et al.*, 2007, Biswas *et al.*, 2010). Therefore, Jordanians having this serotype are potentially at a higher risk of developing *S. mutans*-induced heart-related complications.

Among the diabetic's group isolates, the CBP gene *cnm* was the most prevalent at 75.3 %, followed by *cbm* at 11.1 %. In contrast, among the non-diabetics group isolates, *cnm* and *cbm*, were identified at the same rate (i.e., 38.1 %). Following entry into the circulation, the attachment of *S. mutans* to the exposed collagen tissue is an important step for the initiation of infective endocarditis. Recent studies in Thailand, Fenland, and Japan had shown that the collagen binding proteins encoded by *cnm* and *cbm*, mediate bacterial binding to type I collagen and enhance bacterial virulence (Nakano *et al.*, 2004, Abranches *et al.*, 2011). These genes were predominantly frequent among k and f serotypes, and less frequently among c, and e serotypes. Furthermore, these genes appear to be common worldwide (Abranches *et al.*, 2009, Nomura *et al.*, 2012). Interestingly, *cnm* and *cbm* were most prevalent with serotypes k among diabetic's group isolates, which is consistent with what was reported by others. However, contrary to the previous reports, *cnm* and *cbm* were also prevalent with serotype c isolates among both study groups. Nonetheless, no statistically significant associations were observed regarding the distribution of each of *cnm* and *cbm* with the various serotypes, which is likely due to the small sample size. Although, the serotype k isolates that express CBPs are associated with infective endocarditis, hemorrhagic stroke, and inhibition of platelets aggregation, nonetheless, the relationship between clinical conditions and *S. mutans* serotype remains unclear, as well as the association between *S. mutans* and systemic disease (Nakano *et al.*, 2011, Aikawa *et al.*, 2012).

A study in Japan, reported the presence of *cnm* among approximately 10 % of isolates (Aikawa *et al.*, 2012). *Cnm* in the current study was identified at a much higher frequency than that in Japan. This difference is likely due to inherent differences in predominant strains and genotypes.

Since the current study utilized a small-sized sample, the researchers suggest performing future larger-scale studies in Jordan to investigate the prevalent *S. mutans* serotypes and genotypes among diabetic and non-diabetic individuals, in addition to subjects diagnosed with infective endocarditis. This would yield more representative data for the Jordanian population, and facilitate the identification of potential associations between the serotypes and virulence genes, in addition to the association with clinical diseases.

5. Conclusions

The diabetics and the non-diabetics groups demonstrated similar prevalence of serotype c, e, and f isolates, but significant differences in prevalence of serotype k isolates. Due to the high prevalence of the serotype k isolates among the two study groups, and the high percentage of isolates harboring CBP virulence genes, the Jordanian population are at the high risk of developing *S. mutans*-related complications, such as infective endocarditis.

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Molecular Characterization of *Echinococcus granulosus* sensu stricto Cysts of Domestic Ruminants in Jordan

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Abstract

The semi-nested PCR system and the mitochondrial cytochrome oxidase subunit 1 (*COX1*) partial gene sequencing were used to identify the species/genotypes of 139 hydatid cyst isolates collected from the livers and/or lungs of twenty-nine indigenous ruminant animals (seventeen sheep, three goats, six cattle and three camels) in Jordan. All the examined hydatid cyst isolates identity were *Echinococcus granulosus* sensu stricto (s.s.) which was known formerly as G1-G3 strains. *COX1* partial gene sequencing of twelve selected isolates of hydatid cysts from various intermediate hosts verified their identity as *E. granulosus* s.s. with sequences published in GenBank databases. A unique DNA sample showed two bands within the *COX1* partial gene PCR amplification. The first one was identical to an already published *E. granulosus* s.s. sequence, while the other band had 78 % identity to *E. granulosus* s.s., and 81 % to *E. vogeli COX1* partial gene sequence with two truncated regions (14 and 32 bp) in both species. The present molecular characterization and identification of the hydatid cyst isolates as *E. granulosus* s.s., from indigenous ruminant animals in Jordan confirmed earlier reports that used morphological, *in vitro* and *in vivo* techniques on the existence of *E. granulosus* s.s. G1 strain as the dominant species/genotype in Jordan.

Keywords: Echinococcosis, *Echinococcus granulosus* s.s., Semi-nested PCR, Mitochondrial *COX1* Gene Sequencing.

1. Introduction

Echinococcosis is a cosmopolitan zoonotic helminthic disease caused by a small tapeworm of the genus *Echinococcus* (Cestoda: Taeniidae) that cycles between canid or felid definitive hosts and various herbivores or rodents as intermediate hosts. Humans are accidental hosts that can be infected with the larval stage of the parasite in various internal organs rendering the disease as a major public health risk. The disease has been recently included by the WHO as part of the neglected zoonosis that warrants the implementation of control programs (Da Silva, 2010; Siracusano *et al.*, 2012; Nakao *et al.*, 2013a). Three different forms of echinococcosis are well-recognized in the intermediate hosts and humans: multilocular or alveolar echinococcosis caused by *E. multilocularis*, polycystic echinococcosis caused by *E. vogeli*, and unilocular or cystic echinococcosis (CE) caused by several *E. granulosus* species/strain genotype complex (Moro and Schantz, 2009; Torgerson, 2014).

Currently, several valid *Echinococcus* species are known to cause CE in various herbivorous animals. These include: *E. granulosus* sensu stricto (s.s.), *E. canadensis*, *E. equinus*, and *E. ortleppi* (Nakao *et al.*, 2007; Mcmanus, 2013; Romig *et al.*, 2015). *E. oligarthra* uses wild felids as definitive hosts and agoutis rodents as intermediate hosts and causes unilocular CE. Moreover, *E. shiquicus* has been verified as a distinct species causing unilocular CE in the Tibetan plateau of China (Xiao *et al.*, 2006), while the nature of cysts caused by *E. felidis* has not been elucidated yet. The latter species uses the lion as a definitive host, but its intermediate host is unknown (Huttner *et al.*, 2008).

Echinococcus granulosus manifests great intraspecific phenotypic variations in relation to the host specificity, adult and larval stage morphology, *in vitro* and *in vivo* development, biochemical composition, antigenicity and pathogenicity (Mcmanus, 2013). These variations are reflected by the genetic makeup of nucleic acid sequences that lead to the appearance and differentiation of several genotypes, genetic variants, strains within this species, some of which have now been raised to the level of new species (Mcmanus, 2013; Nakao *et al.*, 2013a; 2013b; Rojas *et al.*, 2014). The current knowledge of the *E. granulosus* sensu lato (s.l.) genotypes and/or species has been reviewed recently. It is well-established now that *E. granulosus* s.l. comprises ten genotypes (G1-G10) (Bowles *et al.*, 1992; Bowles and Mcmanus, 1993a, 1993b; Bowles *et al.*, 1994; Scott *et al.*, 1997; Lavikainen *et al.*, 2003). Accordingly, *E. granulosus* s.l. has been revised to include

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several species that correspond to the previously identified genotypes as follows: *E. granulosus* s.s. (G1- cosmopolitan sheep strain, G2- Tasmanian sheep strain, and G3- buffalo strain), *E. equinus* (G4- sporadic horse strain), *E. ortleppi* (G5- sporadic cattle strain), *E. canadensis* (G6- sporadic camel strain, G7- sporadic pig strain, G8- northern arctic cervid strain, G10- Fennoscandanian cervid strain). As for the G9 (Poland swine strain), its status is still uncertain (Mcmanus and Thompson, 2003; Mcmanus, 2013; Nakao *et al.*, 2013a; 2013b; Rojas *et al.*, 2014; Romig *et al.*, 2015).

Several previous studies indicated that the cosmopolitan common sheep/dog strain (G1 genotype) is the dominant strain in Jordan (Al-Qaoud *et al.*, 2003a; Yanagida *et al.*, 2012). However, other *E. granulosus* strains were reported in Jordan. These include: the horse and camel strains. The horse strain was identified in donkeys using *in vitro* parasite development (Hijjawi *et al.*, 1992), *in vivo* secondary hydatid cyst development in mice (Al-Abbasi and Abdel-Hafez, unpublished data), as well as the RAPD-PCR and RFLP-PCR techniques (Al-Qaoud *et al.*, 2003a). The existence of a camel strain was indicated using morphological criteria of metacestode protoscolices (PSCs) (Said *et al.*, 1988). The present study aimed at the identification and molecular characterization of *E. granulosus* metacestode stage infecting indigenous domestic ruminants in Jordan using semi-nested PCR and partial sequencing of the mitochondrial *COX1* gene.

2. Materials and Methods

2.1. Sample Collection and PSCs Isolation

Some 139 unilocular hydatid cysts were collected from the livers (L) and/or lungs (g) of twenty-nine indigenous livestock animals slaughtered in four different abattoirs located in the cities of Irbid, Ramtha, Jerash (northern Jordan) and Amman (central Jordan) (Table 1). Both fertile and sterile hydatid cysts were isolated from the livers and/or the lungs of indigenous sheep, goats, cattle and camels. Protoscolices (PSCs) and/ or the germinal layer tissues (GL) were isolated under aseptic conditions from each single cyst within six hours after the death of the host as described previously (Nasrieh and Abdel-Hafez, 2004).

2.2. DNA Isolation

DNA was isolated from eighty-four PSCs of fertile cysts and fifty-five germinal layer tissues of sterile cysts (Table 1). PSCs DNA was isolated according to Dinkel *et al.* (1998) using phenol-chloroform-isoamyl alcohol (25:24:1) and ethanol precipitation protocol. Precipitated DNA was re-suspended in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.6) and stored at -20°C for further use. However, the GL tissue DNA isolation was performed using the Cetyl Trimethyl Ammonium Bromide (CTAB) protocol with minor modifications. Primarily, 0.3g GL tissue was digested in liquid nitrogen using small mortar and pestle, and then the mixture was re-suspended in warmed 2-mercaptoethanol/CTAB extraction buffer, and then the plant extraction protocol was followed (Richards *et al.*, 2001).

Table 1. Number of infected animal hosts and hydatid cyst isolates used in the present study, with information on their location and type. DNA samples were extracted from the PSCs of fertile cysts and the germinal membrane of sterile cysts.

Total No. of cysts	No. of sterile cysts	No. of fertile cysts	No. of cyst isolates from		No. of animals	Animal type
			Lung	Liver		
91	15	76	26	65	17	Sheep
22	18	4	1	21	3	Goats
13	13	0	12	1	6	Cattle
13	9	4	10	3	3	Camels
139	55	84	49	90	29	All

2.3. Semi-nested Polymerase Chain Reaction (PCR)

To genotype the prepared DNA samples from hydatid cyst isolates, the semi-nested PCR technique as described by Dinkel *et al.* (2004) was adopted with a minor modification (Figure 1). All PCRs target sequences in this system belong to the mitochondrial 12S rRNA gene, but another set of primers specific to the cytochrome b (Cytb) gene were added to each reaction (as multiplex) to serve as control for the DNA and PCR system.

Initially, the PCR reaction was performed to all extracted DNA samples in order to discriminate *E. granulosus* s.s. from the *E. ortleppi* and *E. canadensis* species. The reaction was performed in a 25µl volume containing 1µl DNA template, 1X green GoTaq reaction buffer, 0.2mM of each dNTP, two units GoTaq DNA Polymerase (all from Promega, USA), 0.4 µM of E.g.ss1for. (5´GTA TTT TGT AAA GTT GTT CTA 3´) and E.g.ss1rev. (5´CTA AAT CAC ATC ATC TTA CAA T 3´) primers, and 0.2 µM of F/Cytb (5´GTC AGA TGT CTT ATT GGG CTG C 3´) and R/Cytb (5´TCT GGG TGA CAC CCA CCT AAA TA 3´) primers. The amplification was carried out after initial denaturation step for four minutes at 94 ºC, for thirty-five cycles as follows: denaturation step for thirty seconds at 94 ºC, annealing step for forty seconds at 54ºC, elongation step for forty seconds at 72 ºC and a final elongation step for five minutes at 72 ºC using TRIO thermoblock (Biometra, Germany).

Samples with the PCR amplification product were considered as *E. granulosus* s.s. species, but for the negative samples, another PCR was carried out using the E.g.cs1for. (5′ ATT TTT AAA ATG TTC GTC CTG 3′) and E.g.cs1rev. (5′ CTA AAT AAT ATC ATA TTA CAA C 3′) primers to verify the presence of *E. ortleppi*/*E. canadensis* species. The positive samples of this primer set were confirmed by semi-nested PCR using the E.g.camel.for. (5′ ATG GTC CAC CTA TTA TTT CA 3′) and E.g.cs1rev. (5′ CTA AAT AAT ATC ATA TTA CAA C 3′) primers for *E. canadensis* species and the E.g.cattle.for. (5′ ATG GTC CAC CTA TTA TTT TG 3′) and E.g.cs1rev. (5′ CTA AAT AAT ATC ATA TTA CAA C 3′) primers for *E. ortleppi* species.

Five microliter of each PCR product was applied to a 1.5 % ethidium bromide stained agarose gel, as well as the 100 bp DNA ladder (AppliChem, Germany). DNA was separated using TBE buffer (89 mM Tris-HCl, 89 mM Boric Acid and 2 mM EDTA.Na_2, pH 8.0) at an electric current of 90 V for forty-five minutes. The produced bands

were visualized on a UV transilluminator (Bioblock Scientific, France).

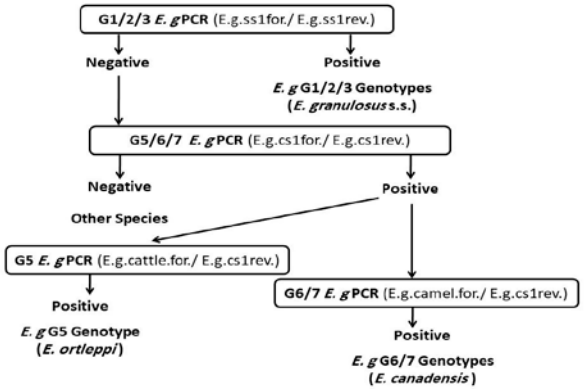

Figure 1. *E. granulosus* s.s., *E. ortleppi* and *E. canadensis* species identification protocol using PCR/semi-nested PCR system (Modified from Dinkel *et al.*, 2004).

2.4. Mitochondrial COX1 Partial Gene Sequencing

Sequencing of a 444 bp segment of the mt *COX1* gene was done for twelve selected samples (1L.9, 3g.3, 11L.2, 13L.3, 14g.6, 16L.3, 18L.4, 19L.3, 22g.3, 23g.4, 26g.2 and 29L.7). The PCR reaction was performed in a 50µL volume containing 1µL DNA template, 1X colorless Taq reaction buffer (Bio-Basic, Canada), 0.4mM of each dNTPs (Promega, USA), 1.5 units Taq DNA Polymerase (Bio-Basic, Canada), 4 mM $MgCl_2$ and 0.2 µM of JB3 (5′ TTT TTT GGG CAT CCT GAG GTT TAT 3′) and JB4.5 (5′ TAA AGA AAG AAC ATA ATG AAA ATG 3′) primers. The amplification was carried out after the initial denaturation step, four minutes at 94 ºC, for thirty-five cycles as follows: denaturation step for thirty seconds at 94 ºC, annealing step for forty seconds at 55 ºC, elongation step for forty seconds at 72 ºC, and a final elongation step for five minutes at 72 ºC (Bowles *et al.*, 1992).

The *COX1* partial gene sense and anti-sense strands were sequenced by ABI 3730XL DNA Analyzer (Applied Biosystems, USA) using BigDye® Terminator V3.1 Sequencing Kit. The sequencing results were analyzed using the BLAST programs and databases of the National Center for Biotechnology Information (NCBI).

3. Results

3.1. All tested samples were E. granulosus s.s. species

Cytb gene target was used to demonstrate the presence of intact DNA within the amplified 139 PSC and GL isolates of hydatid cyst samples. All PCR results were positive for Cytb gene amplification (the 612 bp upper band, Figure 2) except for one sample (sample 15g.1, Fig. 2, lane 16). Moreover, PCR result using mt 12S rRNA gene of the first set of primers (E.g.ss1for./ E.g.ss1rev.) was positive for all samples, which indicates that all the isolated samples were identified as *E. granulosus* s.s. (254 bp, Figure 2). A PSC isolate from camel cysts collected from Iraq in 2003 was used as positive control for *E. canadensis*. DNA from this isolate was negative for the first primer set (absence of 254 bp band, Figure 2, lane 18), but showed positive PCR result for the *E. ortleppi/ E.canadensis* primers (254 bp band, Figure 2, lane 19). Further semi-nested PCR showed that this isolate was positive for the *E. canadensis* set primers (171 bp band, Figure 2, lane 20). None of the isolates from Jordan tested here was positive using the second set of primers (E.g.cs1for./ E.g.cs1rev.).

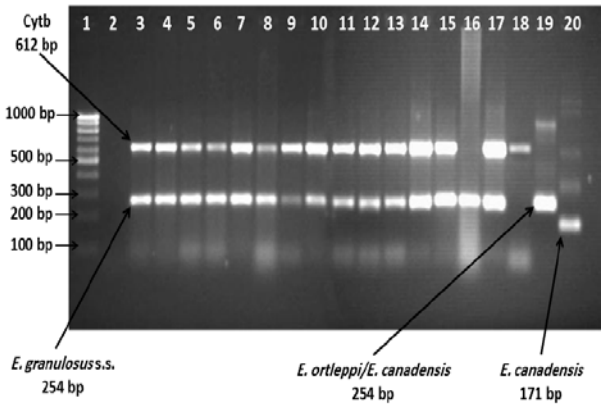

Figure 2. PCR analysis of DNA samples extracted from protoscolices and laminated membranes of selected hydatid cysts from sheep, goat, cattle and camels from Jordan. DNA was amplified using primers specific for 12S rRNA (254 bp lower band) and Cyt b (612 bp upper band) genes. Lane 1: 100 bp DNA marker, lane 2: PCR -ve control, lanes 3 to 17 represent samples from sheep, goat, cattle and camel obtained from liver or lung hydatid cysts, lane 18: 12S rRNA *E. granulosus* s.s. primers -ve control, lane 19: 12S rRNA *E. ortleppi*/*E. canadensis* primers +ve control, and lane 20: 12S rRNA *E. canadensis* primers +ve control.

3.2. Mitochondrial COX1 partial gene sequencing matched the already published sequences in the GenBank

The selected samples representing twelve cysts originated from the lungs and livers of twelve animals were sequenced for the *COX1* partial gene sense and antisense strands. Figure 3 shows the PCR amplification product of the 444 bp of *COX1* partial gene. One sample (13L.3, lane 11) showed a smaller band size. Further analysis of this sample and another one from the same animal (13L.9) revealed the presence of the 444 bp band and an extra band of about 400 bp (Figure 4). All the twelve samples were sequenced including the 400 bp bands of the 13L.3 and 13L.9 samples. Using the BLAST programs and databases of the NCBI, the 444 bp amplification product sequences were compared with *E. granulosus COX1* partial gene sequence published from Jordan sheep/G1 genotype deposited in the EMBL GenBank databases as AB688598.1 (Yanagida *et al.*, 2012). Among the twelve hydatid cysts *COX1* partial gene sequences, four polymorphisms with single-base pair substitution occurred in five DNA samples. Three of them were C to T and one was T to C nucleotide substitution. The other seven DNA samples were completely identical to the reference sequence.

Samples 13L.3 and 13L.9 400 bp band sequence showed higher variations when compared to the original *E. granulosus* s.s. *COX1* partial gene sequence (AB688598.1). The sample sequence showed two main deletions of 14 bp and 32 bp sizes (Figure 5). The sequence shows forty mismatched nucleotides with a max identity of 78 % (311/397 bp) compared to *E. granulosus* sheep/G1 genotype sequence, while twenty-seven mismatched nucleotides were counted compared to other

published *COX1* partial gene sequences, *E. vogeli* (accession number JX315616.1) with a higher identity (81 %, 323/397 bp). The two main deletions were found at the same sites in both reference *E. granulosus* and *E. vogeli* sequences. However, when both *E. granulosus* and *E. vogeli* sequences were aligned, 91 % max identity occurred with thirty-five mismatched nucleotides (Figure 5).

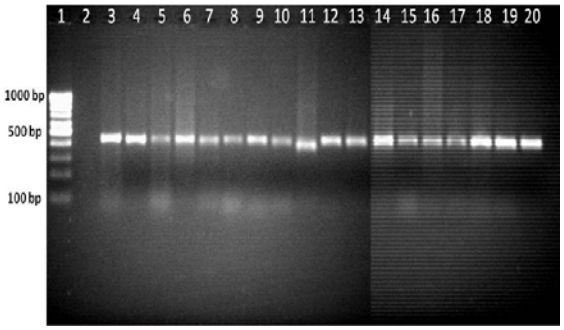

Figure 3. PCR amplification of 444 bp *COX1* for selected hydatid cyst samples from Jordan. Lane 1: 100 bp DNA marker, lane 2: PCR -ve control, lanes 3 to 20 represent samples: 1L.9, 3g.5, 4L.1, 7g.4, 9L.4, 10g.2, 11L.2, 12L.2, 13L.3, 14g.6, 16L.3, 18L.4, 19L.3, 22g.3, 23g.4, 24L.4, 26g.2, and 29L.7, respectively.

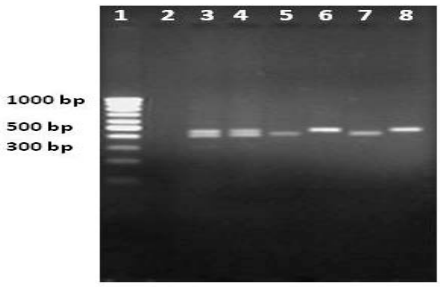

Figure 4. PCR amplification of *COX1* gene for two hydatid cyst samples. Lane 1: 100 bp DNA marker, lane 2: -ve control PCR product, lanes 3&4: *COX1* partial gene PCR amplification product for sample 13L.3 and 13L.9, respectively. Lane 5&7: the purified 400 bp band of the 13L.3 and 13L.9 samples, respectively. Lanes 6&8: the purified 444bp band of the 13L.3 and 13L.9 samples, respectively.

```
E.granulo 1-60      GTGTTGATTTTGCCTGGATTTGGTATAATTAGTCATATTTGTTTGAGTATTAGTGCTAAT
13L.3               GTGTTAATTTTGCCAGGGTTTGGCATAATTAGTCATATTATGTTTGAGAATTAGTGCTAAT
E. vogeli           GTGTTAATTTTGCCTGGGTTTGGTATAATTAGTCATATTATGTTTGAGGGTTAGTGCTAAT

E.granulo 61-120    TTTGATGCGTTTGGGTTCTATGGGTTGTTGTTTGCTATGTTTTCTATAGTGTGTTTGGGT
13L.3               TTGGATGCATTTGGGTTTTATGGGTTGTTATTTGCTATGTTTTCTATAGTAT-------A
E. vogeli           TTGGATGTGTTTGGGTTTTATGGGTTATTGTTTGCTATGTTTTCTATAGTATGTTTAGGA

E.granulo 121-180   AGCAGGGTTTGGGGTCATCATATGTTTACTGTTGGGTTGGATGTGAAGACGGCTGTTTTT
13L.3               -------GTTTGAGGGCATCATATGTTTACTGTTGGGTTAGATGTTAAGACGGGTGT-TTT
E. vogeli           AGTAGGGTTTGAGGGCATCATATGTTTACTGTTGGGTTGGATGTGAAGACGGCTGTKTTT

E.granulo 181-240   TTTAGCTCTGTTACCTATGATTATAGGGGTTCCTACTGGTATAAAGGTGTTTACTTGGTT
13L.3               TTTAATTCTGTTAGCTATGATTATAGGGTGTTCCTACGGGTATAAAGGTTTTAACTTGGTT
E. vogeli           TTTAGTTCTGTTACCTATGATTATAGGGGTTCCTACTGGTATAAAGGTGTTTACTTGGTT

E.granulo 241-300   ATATATGTTGTTGAATTCGAGTGTTAATGTTAGTGATCCGGTTTTGTCATGCGGTTGTTTC
13L.3               GTACATGCTGTTGAATTTGAGTGTGAATAAGAGTGATCCGGTTT-----------------
E. vogeli           GTATATGTTGTTGAATTCGAGTGTGAATAAGGGTGATCCGGTTTTATGGTGAGTGGTTTC

E.granulo 301-360   TTTTATAGTGTTGTTTACGTTTGGGGGAGTTACGGGTATAGTTTTGTCTGCTTGTGTGTT
13L.3               ----------------ACTTTTGGTGGTATGACTGGTATAGTTTTGTCTGCATGTGTATT
E. vogeli           TTTTATAGTGTTGTTTACGTTTGGTGGTGTTACTGGTRTAGTTTTGTCTGCTTGTGTATT

E.granulo 361-396   AGATAATATTTTGCATGATACTTGGTTTGTGGTGGCT
13L.3               GGATAATGTTTTGCATGATACTTGGTTTGTGGTGGCT
E. vogeli           GGATAATGTTTTGCATGATACTTGGTTTGTGGTAGCT
```

Figure 5. DNA sequence alignment of the 400 bp segments of the 13L.3 *COX1* gene compared with *E. vogeli COX1* partial gene (accession number JX315616.1) and *E. granulosus COX1* partial gene (accession number AB688598.1) sequences. Gray blocks represent mismatched base pairs, while bold nucleotides represent three stop codons within the 13L.3 sample sequence according to the normal reading frame (without deletions).

4. Discussion

In the present study, semi-nested PCR system and mt*COX1* gene sequencing techniques were used to screen hydatid cysts collected from infected livestock in Jordan. The semi-nested PCR system used by Dinkel *et al.* (2014) to characterize the genotypes of *E. granulosus* with high specificity (100 %) and sensitivity (0.25 pg of DNA) was adopted successfully in this study. The system was comparable with other protocols of RFLP-PCR and sequencing of mt*COX1* and *NDI* genes (Dinkel *et al.*, 2004), and has been utilized by several research groups in the region for this purpose (Pour *et al.*, 2011; Elhag *et al.*, 2013; Hammad *et al.*, 2018). Using Dinkel's semi-nested PCR system, all the collected samples from Jordanian livestock in the present study proved to conform to *E. granulosus* (s.s.). All samples were distinctly different in their mt 12S rRNA gene profile from that of *E. canadensis* which characterized the camel control samples originating from Iraq (Figure 2).

However, *E. canadensis* species (camel G6 genotype) was isolated from sheep, goats, cattle, camels and humans in Sudan, Tunisia, Iraq, Iran, Egypt and Kenya (Dinkel *et al.*, 2004; Shahnazi *et al.*, 2011; Rajabloo *et al.*, 2012; Elhag *et al.*, 2013; Boufana *et al.*, 2014; Khalifa *et al.*, 2014; Pestechian *et al.*, 2014; Amer *et al.*, 2015; Hammad *et al.*, 2018). In the present study, none of the thirteen collected camel cysts were found to be infected with *E. canadensis*. This may be explained by the limited importation of exogenous camels in Jordan, the use of mixed livestock herds and the dominancy of the highly fertile *E. granulosus* sheep G1/dog cycle.

To verify the PCR results, twelve samples representing twelve infected intermediate hosts were selected for mt*COX1* partial gene sequencing. Sequencing of the mtDNA markers has more power than nuclear DNA in reconstructing the phylogenetic relationships among the closely-related species, and proved to be useful as an important genetic marker for the differentiation and studying of the population genetic structure of *E. granulosus* genotypes (Nakao *et al.*, 2013a; Sharma *et al.*, 2013). Mitochondrial *COX1* gene sequencing has been applied successfully in order to distinguish different *Echinococcus* species and genotypes (Bowles *et al.*, 1992; Abushhewa *et al.*, 2010; Casulli *et al.*, 2012; Nakao *et al.*, 2013a; Amer *et al.*, 2015; Abbas *et al.*, 2016; Debeljak *et al.*, 2016; Kandil *et al.*, 2016).

Although the identity of the 400 bp band sequence with *E. vogeli* was higher than that with *E. granulosus* (3 % difference, 12 bp), the life cycle and distribution of *E. vogeli* exclude the possibility of its existence in hosts indigenous to Jordan. *E. vogeli* typically uses the South American bush dog (*Speothus venaticus*) as a wild definitive host and pacas and agoutis as intermediate hosts (D'alessandro and Rausch, 2008). Taking into consideration the fact that both self and cross fertilization can occur in *Echinococcus* species (Haag *et al.*, 1999), the appearance of two bands in the 13L.3 and 13L.9 *COX1* partial gene PCR products may reveal the presence of two *COX1* gene alleles since the DNA source is genomic. Moreover, such finding may be explained by the presence of *pseudogenes*. *Pseudogenes* reported in mitochondrial and ITS genes of parasitic helminths, including

Echinococcus (Van Herwerden *et al.*, 2000; Obwaller *et al.*, 2004). *Pseudogenes* have been defined as "mitochondrial-like sequences found in the nuclear genome" (Lavikainen *et al.*, 2008) or as "sequences containing internal stop codons and/or deletions associated with frame shifts (Obwaller *et al.*, 2004; Lee *et al.*, 2007). Both definitions conform to the present findings since the DNA was genomic and internal stop codons and deletions have occurred.

Moreover, variations among parasitic helminths mt*COX1* gene sequences have been documented in several studies. A study by Obwaller *et al.* (2004) reported on a single base deletion and an internal stop codon in the *COX1* gene sequences of four *Echinococcus* species with a variation level of 2.45%. Also, Lee *et al.* (2007) reported that differences in the *COX1* gene sequences ranged from 1-121 bp in trematodes and 0-28 bp in cestodes. The largest gap was 121 bp between *Heterophyes nocens* and *Plagiorchis muris* in trematodes, and 28 bp between *Diphyllobthrium latum* and *Taenia taeniaeformis* in cestodes. In addition, Lavikainen *et al.* (2008) reported on the high intraspecific nucleotide sequence variations in the *COX1* gene within both *T. polyacantha* and *T. taeniaeformis* species.

In conclusion, semi-nested PCR system and *COX1* partial gene sequencing are useful for the characterization of *Echinococcus* specimens. Moreover, molecular analysis of the cysts of sheep, goats, cattle, and camels collected from four different abattoirs in Jordan proved the dominance of *E. granulosus* s.s. (G1-G3 strains). This finding supports earlier studies in Jordan regarding the existence of the former genotype of *E. granulosus* s.s. G1 strain as the dominant species/genotype in Jordan (Abdel-Hafez and Kamhawi, 1997; Al-Qaoud *et al.*, 2003a; Yanagida *et al.*, 2012). As the cosmopolitan sheep/dog G1 strain of *E. granulosus* s. s. is responsible for 88.44 % of the human CE worldwide (Rojas *et al.*, 2014), the dominance of this species in Jordan as documented in the present study explains well the high endemicity of the disease in Jordan (Al-Qaoud *et al.*, 2003b). Accordingly, and as different strains or species of *E. granulosus* may exhibit variations in epidemiology, transmission patterns as well as sensitivity to chemotherapeutic agents and drugs (Siracusano *et al.*, 2012; Rojas *et al.*, 2014), any control program to be envisaged for CE in Jordan must take into consideration that *E. granulosus* s.s. is the dominant species or strain in the country.

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Synthesis, Molecular Docking and Antioxidant Evaluation of Benzylidene Ketone Derivatives

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Abstract

Four benzylidene ketone derivatives were synthesized by reacting 4-nitrobenzaldehyde with several ketone derivatives. The synthesized compounds A, B, C and D had shown antioxidant activity against deoxyribose degradation, while compound C showing the highest activity. The molecular docking simulation indicated that the superior activity of compound C among other compounds can be attributed to the alkyl elongation at ketone chain.

Keywords: Antioxidants, Molecular docking, Deoxyribose degradation, Benzylidene ketone derivatives.

1. Introduction

Oxidative stress maybe defined as the imbalance between the production of free radicals and the ability of the organism to neutralize its action by antioxidant systems (Pisoschi and Pop, 2015). Free radicals are unstable atoms, molecules or ions with unpaired electrons that are chemically reactive with other molecules (Carocho and Ferreira, 2013) such as reactive oxygen species (ROS), reactive nitrogen species (RNS), and reactive sulfur species (RSS). Studies have shown that free radicals are associated with many chronic health problems: such as Parkinson, Alzheimer, cancer, cardiovascular and inflammatory diseases (López-Alarcón and Denicola, 2013, Maulik *et al*., 2013, Toda, 2011). Thus, to prevent such serious medical problems, the elaboration of novel synthetic compounds is significant in the field of modern drug design and discovery.

Free radical formation is mainly controlled naturally by various beneficial compounds known as antioxidants. The terminology describing the actions of antioxidants is unfortunately not completely clear because there are various types of antioxidants (Carocho and Ferreira, 2013). Halliwell defined antioxidants as ‘‘any substance that delays, prevents or removes oxidative damage to a target molecule’’(Halliwell, 2011), while Khlebnikov defined them as ‘‘any substance that directly scavenges ROS or indirectly acts to up-regulate antioxidant defenses or inhibit ROS production’’(Khlebnikov *et al*., 2007).

The screening studies for antioxidant properties of medicinal and edible plants have been performed progressively over the last decades in the hope of finding an effective therapy for numerous modern diseases and also postponing aging symptoms (Halliwell, 2008).

Literature has proved that Curcumin, the active constituent of Curcuma species plant (Masuda *et al*., 1992) has a wide range of physiological activity. Such activities include but are not limited to: antioxidant, anti-inflammatory, anticancer, chemopreventive, antibacterial, antifungal, antiparasitic, antiviral and antihistaminic activities (Kocaadam and Şanlier, 2017).

The modification of the curcumin molecule structure has been studied due to its instability toward light, pH, temperature, and its poor pharmacokinetic profiles. It was intended to enhance the stability and absorption of curcumin when administered orally. Sardijman *et al*. have synthesized many curcumin analogues, one of them was 2,5-*bis*(4'-*hydroxy*-3'-methoxybenzylidene) cyclopentanone (pentagamavunone-0) which were benzylidene ketone derivative (Sardjiman *et al*., 1997). Those compounds have been examined as antioxidant (Reksohadiprodjo, 2004), anti-inflammatory (Masuda *et al*., 1992), and antitumor (Youssef *et al*., 2004).

Deoxyribose degradation will produce malonaldehyde that is identified by red color of the thiobarbituric acid (TBA) complex. A few of benzylidene ketone derivatives have been synthesized and shown the antioxidant properties by inhibiting deoxyribose degradation (Handayani and Arty, 2008). Previously, our studies reported the use of a molecular docking technique as a valuable tool to quickly study the drug-target intermolecular interactions (. Al-Najjar *et al*., 2017, Shakya *et al*., 2016, Mohseni *et al*., 2016, Muchtaridi *et al*., 2014). Several studies highlighted a specific tyrosine kinase enzyme (Src family tyrosine kinase Hck) with the

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potential of being used as a therapeutic target for antioxidant compounds *(Singh, 2015, Sassi et al*., 2017). In the present study, four benzylidene ketone derivatives that contain a nitro group in an aromatic ring have been synthesized, and evaluated for their antioxidant properties via deoxyribose degradation inhibition mechanism, as well as, studying the intermolecular interactions by AutoDock 4.

2. Materials and Methods

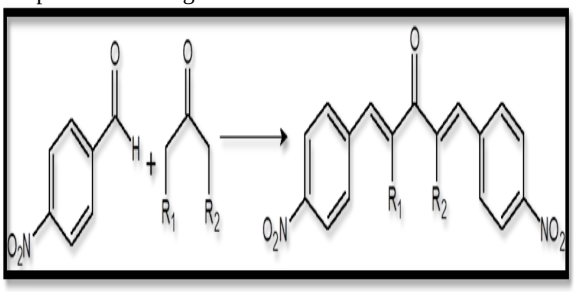
2.1. Chemical Synthesis

4-nitrobenzaldehyde; cyclopentanone; cyclohexanone; acetone; 2-pentanone; 2-heptanone; curcumin (Figure 1); sodium hydroxide; ethanol; chloroform; methanol; ethyl acetate; water; silica F_{254}; dimethyl sulphoxide-d_6; $CDCl_3$; KBr pellet; column C_{18}; 2-deoxyribose; phosphate buffer pH 7.4; hydrogen peroxide; ferrous sulphate; thiobarbituric acid; phosphoric acid. All materials used were analytical grade that were obtained from Sigma Aldrich.

UV_{254} lamp, UV spectrophotometer (Perkin Elmer Lambda 25 UV-VIS spectrophotometer), NMR spectrometer (Bruker Avance NMR 400 MHz); GC-MS (Turbo Mass EI-MS), safety cabinet, and micropipette were used.

2.2. General Synthetic Procedure of Benzylidene Ketone

A 0.066 mole of 4-nitrobenzaldehyde was mixed with several ketone derivatives (0.033 moles) as shown in the reaction scheme 1 (Thirunarayanan and Ananthakrishna Nadar, 2006, Amoozadeh *et al*., 2010). The mixture was stirred homogeneously at 25°C-30°C. 15 mL of ethanol was mixed homogenously with 10 mL of NaOH 10% solution into a separated glass beaker. Half of the second mixture was added to the first mixture, and was then stirred homogenously for fifteen minutes. Thereafter, the remaining second solution was added and stirred gently for thirty minutes. The reaction mixture was kept at room temperature overnight.



Scheme 1: General synthesis route.

The mixture was obtained in an oily form and then dropped wisely using HCl 10 % until the neutral pH was obtained and the crystal started to grow. The mixture was filtered, and the precipitate was rinsed with water and ethanol 70 %. Finally, the crystal was recrystallized using ethanol 95 %; a reddish to deep brown crystal was obtained as a product as in Figure 1.

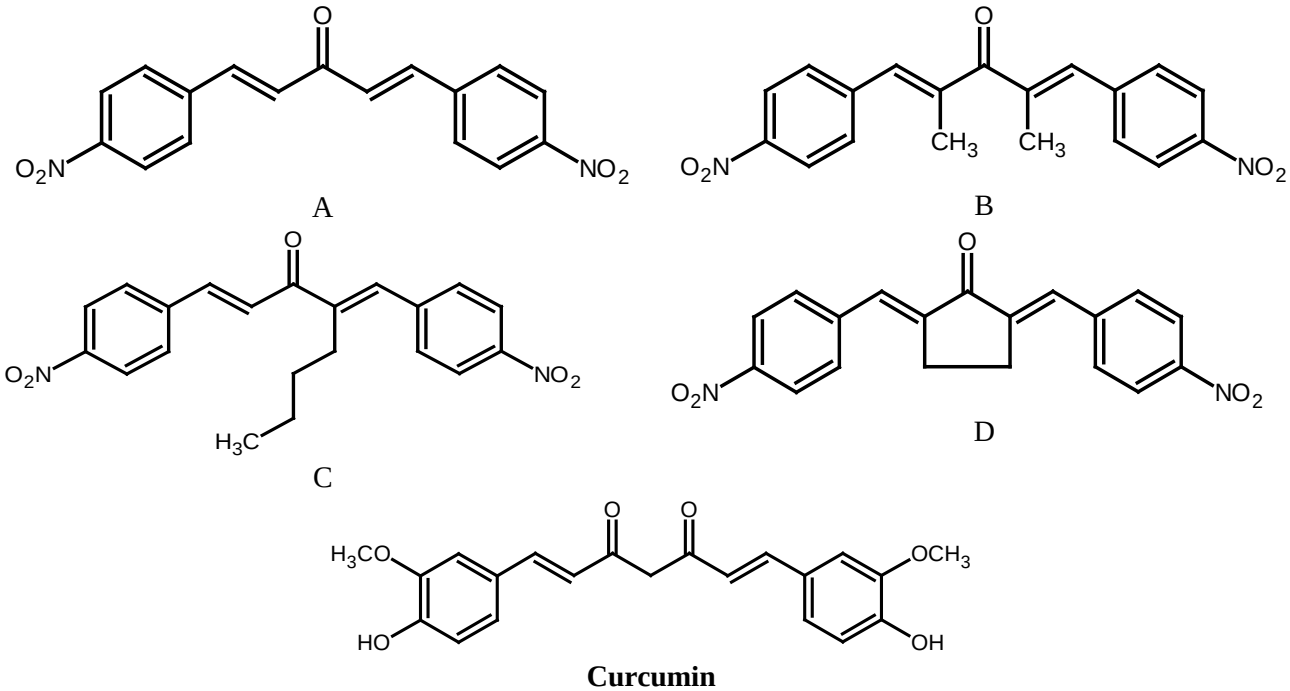


Figure 1. 2D Chemical structures of synthesized compounds and curcumin (positive control).

2.3. Analysis of Physicochemical Properties, Purity and Molecule Structure

The product was examined by thin-layer chromatography for three times using silica gel F_{254} plate with methanol: chloroform (2:8) solvent. The spot was detected by a UV lamp and obtained the R_f. The maximum wavelength was measured by dissolving sufficient amounts of the product in ethanol, and was measured by UV-VIS spectrophotometer. Furthermore, a sample of the product was dissolved in methanol, and was then injected to Gas Chromatography-Mass Spectrometer (Gas-MS) instrument. The condition of GC-MS was listed as follows: Oven: Initial temp 50°C for two minutes, ramp 10°C/min to 250°C, hold eight minutes, Inj = 250°C, Volume = 2 µL, Split = 10:1, Carrier Gas = He, Solvent Delay = 2.00 min,

Transfer Temp= 20^0C, Source Temp = 250^0C, Scan: 28 to 500Da, Column 30,0m x 320µm, TR WAX MS.

Finally, the chemical structures of the synthesized products were confirmed by NMR by dissolving a sufficient amount of the product in DMSO-d_6.

1,5-bis (4-nitrophenyl)penta-1,4-dien-3-one (A)

Brown powder; Yield 52 %; R_f 0.82 (MeOH : water = 8:2); λ_{max} 301 nm; ^{1}NMR: δ 6.88 (2H, d, *J* = 16.6 Hz), 7.84 (4H, ddd, *J* = 8.7, 2.3, 0.5 Hz), 7.93 (2H, d, *J* = 16.6 Hz), 8.08 (4H, ddd, J = 8.7, 1.9, 0.5 Hz).

2,4-Dimethyl-1,5-bis(4-nitrophenyl)penta-1,4-dien-3-one (B)

Brown powder; Yield 62 %; R_f 0.82 (MeOH : water = 8:2); λ_{max} 285 nm; ^{1}NMR: δ 1.86 (6H, s), 7.80-7.87 (6H, ddd, 7.84 (ddd, *J* = 8.7, 2.3, 0.5 Hz), 7.83 (s)), 8.07 (4H, ddd, *J* = 8.7, 2.1, 0.5 Hz)

1-(4-nitrophenyl)-4-[(4 nitrophenyl) methylidene] oct-1-en-3-one (C)

Reddish Brown powder; Yield 44 %; R_f 0.85 (MeOH : water = 8:2); λ_{max} 275 nm; ^{1}NMR: δ 0.88 (3H, t, *J* = 6.5 Hz), 1.31 (2H, h, *J* = 6.5 Hz), 1.64 (2H, tt, *J* = 7.1, 6.5 Hz), 2.51 (2H, t, *J*=7.1 Hz), 7.00 (1H, d, *J* = 16.5 Hz), 7.80-7.87 (4H, 7.83 (ddd, *J* = 8.7, 2.3, 0.5 Hz), 7.84 (ddd, *J* = 8.7, 2.2, 0.5 Hz)), 7.89-7.98 (2H, 7.89(s), 7.94 (d, *J*= 16.5 Hz)), 8.04-8.11 (4H, 8.07 (ddd, *J* = 8.7, 2.1, 0.5 Hz), 8.08 (ddd, *J* = 8.7, 1.9, 0.5 Hz)

2,5-bis[(4-nitrophenyl)methylidene]cyclopentan-1-one (D)

Dark Brown powder; Yield 66 %; R_f 0.80 (MeOH : water = 8:2); λ_{max} 334 nm; ^{1}NMR: δ 2.89 (4H, ddd, *J* = 12.3, 8.1, 4.1 Hz), 7.80-7.87 (6H, 7.83 (ddd, *J* = 8.7, 2.3, 0.5 Hz), 7.82 (s)), 8.07 (4H, ddd, *J* = 8.7, 1.9, 0.5 Hz).

2.4. 2.4 Molecular Modeling

The following software packages were utilized in the present research:

- ChemSketch ACD labs release 12.01 (www.acdlabs.com) (Advanced Chemistry Development, 2016).
- Autodock 4.2, Scripps Research Institute (http://autodock.scripps.com) (Morris *et al.*, 2009).

Molecular Docking: molecular docking simulations can be used to study and understand the intermolecular interactions between the synthesized compounds and the molecular target. Molecular docking simulation was performed on the X-ray crystal structures of protein tyrosine kinase (PDB code: 2HCK) (Sicheri *et al*., 1997) utilizing AutoDock 4.2 software (Morris *et al*., 2009). The protein crystal structure was initially prepared by merging all of the non-polar hydrogens and removing water molecules. Both Gasteiger and Kollman united atom charges were added to ligands and enzyme, respectively. The ADDSOL utility embedded in Autodock 4.2 was used to assign the atomic solvation parameters. The grid calculation was performed using Autogrid4 program, in which a box dimension of 22.5 Å and grid spacing of 0.375 Å parameters were set. The Lamarckian genetic algorithm (LGA) was used as a global optimizer and energy minimization for docking simulation.

2.5. Evaluation of Antioxidant Activities

2.5.1. Preparation of Sample Solution

A sufficient amount of the synthesized compounds was dissolved in DMSO to create a concentration series, i.e. 50 ppm, 100 ppm, 250 ppm, 500 ppm, and 1000 ppm. Moreover, curcumin was used as a positive control with the same series concentration.

2.5.2. Antioxidant Assay Using Deoxyribose Degradation Method.

This method is based on the determination of malondialdehyde (MDA), a degradation product of 2-deoxyribose, by the measurement of the condensation product with thiobarbituric acid (TBA). Typical reactions for the blank solution were started by the addition of 0.5 mM $FeSO_4$ to solutions (0.5 mL final volume) containing 5 mM 2-deoxyribose, 10 mM phosphate buffer (pH 7.2) and 2 mM H_2O_2. Reactions were carried out for ten minutes at room temperature (24–25 °C) and were stopped by the addition of 0.5 mL 4 % phosphoric acid v/v followed by the addition of 0.5 ml 1%TBA in 50 mM NaOH solution. After boiling for fifteen minutes, solutions were allowed to cool down to room temperature, and the absorbance was measured at 530.4 nm. The same procedure was repeated by adding a concentration series of the sample after the addition of H_2O_2.

The percentage inhibition was calculated by the following equation:

$$I(\%) = \frac{A_{blank} - A_{sample}}{A_{blank}} \times 100\%$$

The IC_{50} value represented the concentration of the compounds that caused 50 % inhibition calculated by using linear regression between the concentration of samples and its % inhibitions.

3. Results and Discussions

Dichloro- diphenyl- phenylhydrazine (DPPH) method is commonly used to determine the antioxidant property based on the free radical scavenging by polyhydroxyl compounds. Because of its resonance effect along the conjugated double bond once it was triggered by free radicals, it would be reactive to scavenge them and form the stable product. The free radical reaction mechanism was presented in figure 2 (Josephy and Mannervik, 2006).

The hydroxyl radical came from iron (II) and hydrogen peroxide, thus it will oxidize polyhydroxy of deoxyribose producing malonaldehyde. The oxidized product can be detected by thiobarbituric acid which is used to carry out the colorimetric reaction. A Pink solution was obtained due to the conjugated double bond elongation of two molecules of TBA and one molecule of malonaldehyde via Claissen Schimdt condensation under acid condition.

Benzylidene ketone had the long conjugated double bond, therefore it would be an inhibitor analogue in the oxidation process of D-ribose by perhydroxy radical (Figure 2) (Josephy and Mannervik, 2006). The absorbance of the pink color was measured using a visible spectrophotometer at the optimum wavelength (530.40 nm).

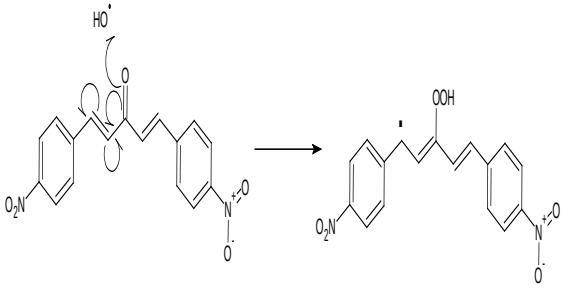


Figure 2. Proposed Mechanism of Hydroxyl Radical Scavenging of Benzylidene Ketone.

The antioxidant activity was calculated as the percentage of the decreasing product absorbance that could prevent the degradation of the 2-deoxyribose compared to the blank. Thus, the more intense the pink color was, the less active the understudy compound was. Using Linear Regression analysis, the IC_{50} of each compound could be determined by extrapolating to the correlation between concentration versus inhibiton percentage (% I). The IC_{50} of four benzylidene ketone derivatives, as well as curcumin are presented in Table 1, in which compound C showed better antioxidant activity than curcumin as displayed lower IC_{50} values.

Table 1. The IC_{50} of synthetic products against curcumin as positive control.

Compounds	IC_{50} (ppm)
A	511.3
B	723.8
C	140.6
D	636.2
Curcumin	527.5

The molecular docking studies have been carried out to evaluate the binding affinity of benzylidene ketone derivatives with protein tyrosine kinases (PDB code: 2HCK). The intermolecular interactions of the actively-docked conformations are identified with all amino acids within 5 Å of the active site. Table 2 shows the docking scores and the estimated free energy of binding (FEB). All the compounds showed comparable energy values with better binding affinity for curcumin. All the compounds bound similarly in the active site as shown in figure 3. As discussed earlier, the compound that showed better antioxidant activity was compound C, thus it will be beneficial to compare the intermolecular interactions with curcumin. Curcumin showed to perform hydrogen bond interactions with Asp404, Lys295, Met341 and Ser345, as shown in figure 4. On the other hand, compound C performed four hydrogen bond interaction with Gln277, Lys295, Met341 and Ser345, as well as hydrophobic interaction with Leu273 and Leu393 as shown in figure 5.

Table 2. Docked energy and FEB for compounds A, B, C, D and Curcumin.

Compound	Docked Energy (Kcal/mole)	Estimated FEB (Kcal/mole)
A	-7.28	-7.53
B	-7.63	-7.86
C	-7.25	-7.84
D	-7.62	-7.79
Curcumin	-8.56	-8.61

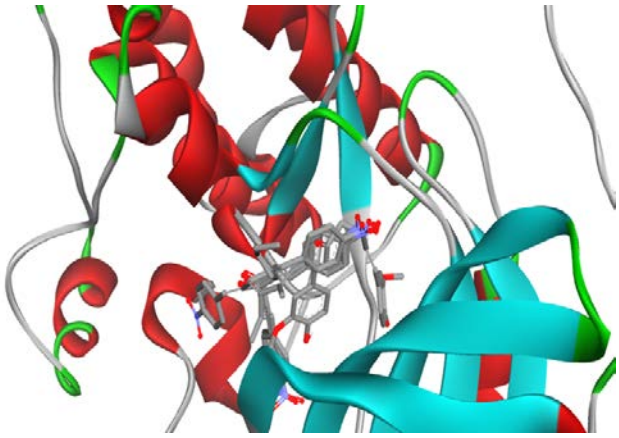
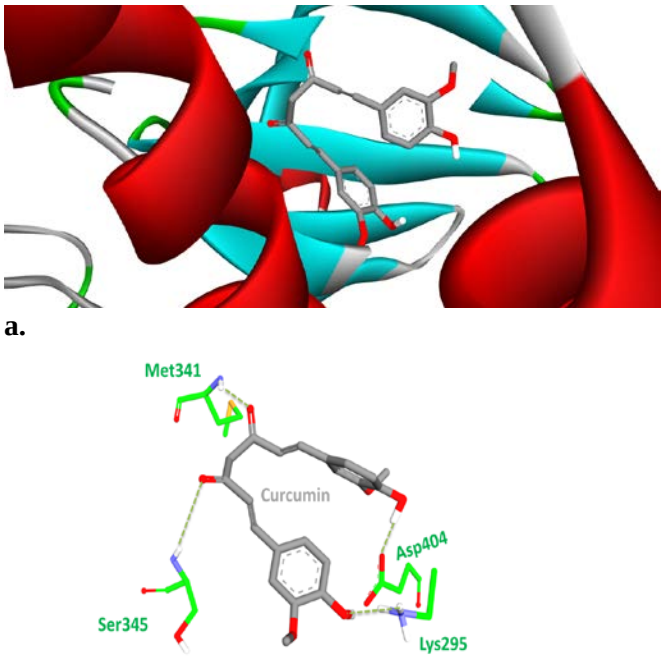


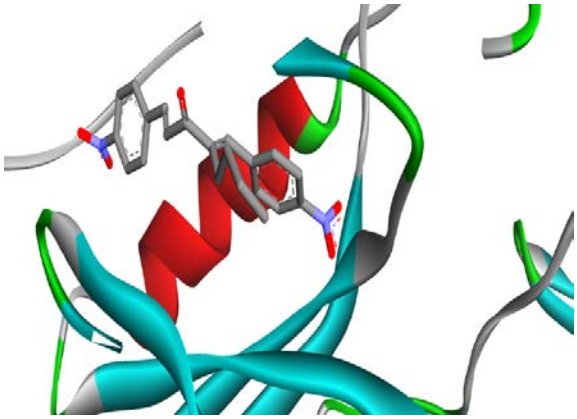
Figure 3. Solid ribbon representation of 2HCK showing the docked compounds (A, B, C, D and curcumin) in the active site.



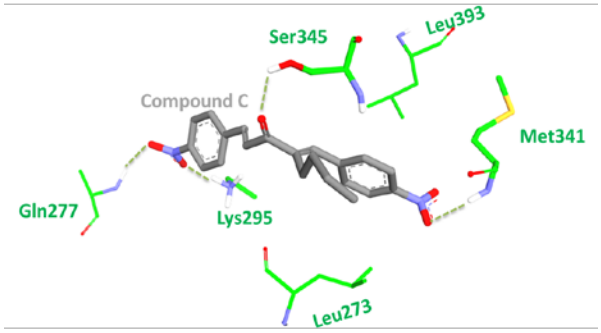
a.

b.

Figure 4. Docked curcumin pose. a. solid ribbon representation of 2HCK with curcumin docked pose. b. stick representation of active site residues that form hydrogen bond interactions (green dashed line) with curcumin.



a.



b.

Figure 5. Docked compound C pose. a. solid ribbon representation of 2HCK with compound C docked pose. b. stick representation of active site residues that form hydrogen bond interactions (green dashed line) with compound C.

4. Conclusion

A synthesis reaction was carried out to synthesize four benzylidene ketone derivatives. All synthetic products had comparable antioxidant activity against deoxyribose degradation with curcumin as positive control. Compound C showed better antioxidant scavenging activity than other compounds. Molecular docking simulation indicated that the superior activity of compound C among other compounds might be attributed to the alkyl elongation at ketone chain. Finally, biological and docking studies may help scientists to design and develop potent and selective antioxidant compounds.

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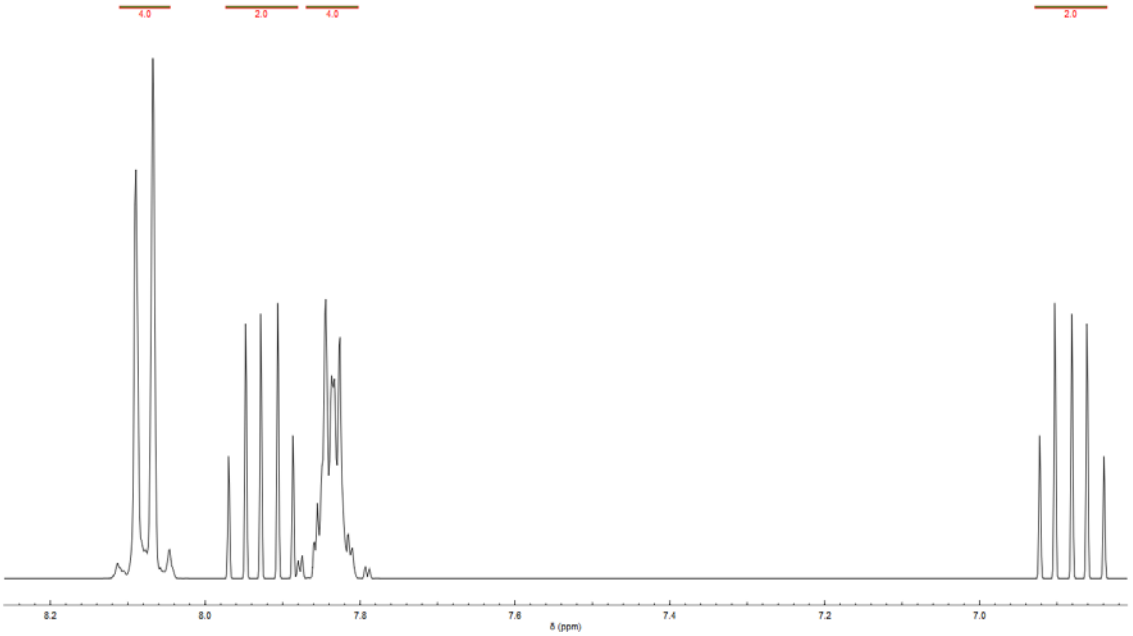
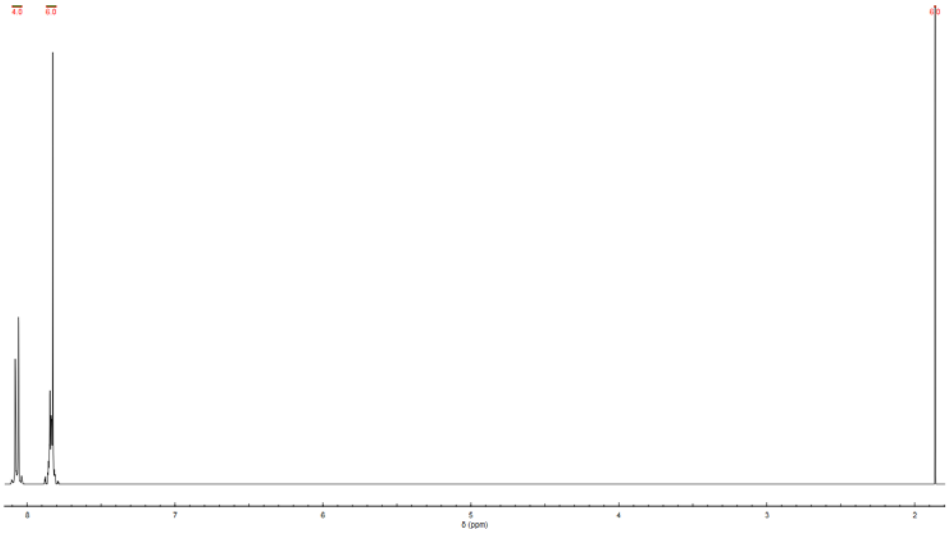
NMR spectra



Compound A



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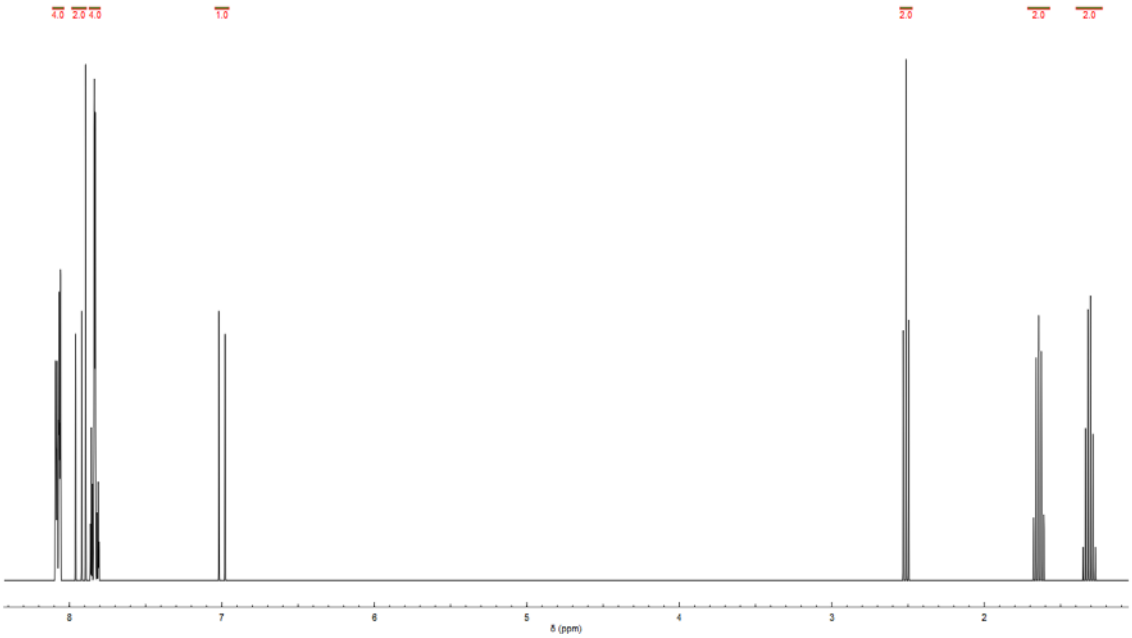

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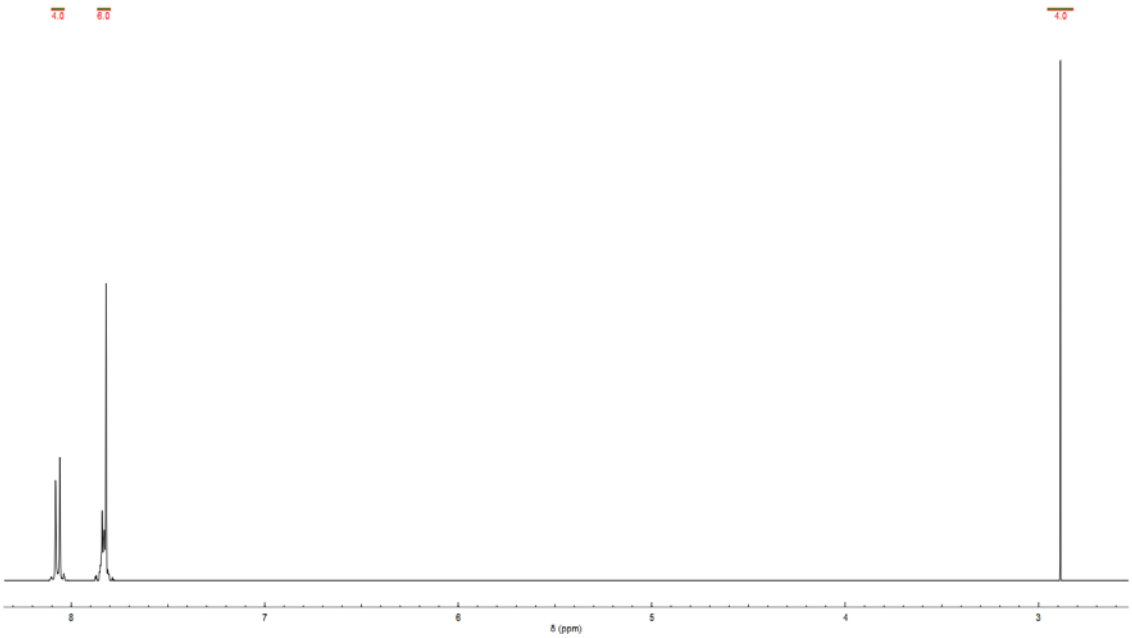

Compound D

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Determination of Antioxidant Properties and the Bioactive Compounds in Wheat (*Triticum aestivum* L.)

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Abstract

The antioxidant property and bioactive compounds' activity of two wheat varieties have been investigated in this research. To conduct this experiment, leaves and seeds of wheat were extracted by methanol and ethanol. For DPPH free radical scavenging assay, plant extracts showed 50 % scavenging ability compared with standard antioxidant (BHT). The results revealed that the Kheri leaf extracts had 2.17, 5.03, 11.74, 25.44, 39.64, 49.41 and 54.34% with various doses which clearly shows that it is capable of scavenging the 50 % DPPH. The Kheri seed extracts showed IC_{50} value of 55µg/mL for scavenging the 50 % DPPH. On the other hand, the Pavon 76 leaf and seed extract showed an IC_{50} value of 80 µg/mL and 90µg/mL at different concentrations. The ethanol extracts of the two tested genotypes had an abstemiously-high antioxidant effect though this effect was not approximate to the synthetic antioxidant (BHT). Moreover, the extracts of various solvents were tested to identify the nature of phytochemical constituents which exposed several bioactive compounds such as alkaloid, flavonoids, phenols, saponins, amino acids and carbohydrates present in trace amount in the case of whole-plant extracts. The antioxidant property in whole grains may be responsible for the health benefits of the whole-grain consumption of wheat.

Keywords: Antioxidant activity, Bioactive compounds, DPPH assay, Phytochemical analysis, *Triticum aestivum.*

1. Introduction

Secondary metabolic compounds found in plants are effective sources of protection against insect attacks, plant diseases and chronic microbial effects. The most important of these bioactive constituents of plants are alkaloids, tannins, flavonoids, cardiac glycosides, steroids and saponins (Nithya *et al.,* 2011; Morshed and Islam, 2015). The edible oils and fats are generally oxidized in the presence of oxygen and light resulting in the formation of reactive oxygen species which have been associated with cancer, cardiovascular diseases, inflation and aging (Siddhuraju and Beeker, 2003). The consumption of oxidized lipids is associated with oxidation of biological membranes, genotoxicity and tocopherol inhibition (Sikwese and Duodu, 2007). Antioxidants encounter the reactive oxygen species, and reduce the risk associated with lipid oxidation. There has been an increasing interest on the part of the food industries and preventive medicines to replace synthetic antioxidants with those of safer and more natural origins as stated by Shahidi (1997) and Chatha *et al.* (2006). Cereals and other crops antioxidants have gained substantial interest over the last few years (Bhattacharjee *et al.,* 2015; Paul *et. al.,* 2017). Fleischman *et al.* (2016) evaluated antioxidant capacity to incorporate wheat bran into an extruded snack. They reported that extrudates made with the addition of red (37.5 %) and purple (37.5 %) bran had higher values compared to the other treatments. They also mentioned that the control, red, and white bran treatments had less antioxidant activity after extrusion compared to purple bran supplemented extrudates. Purple and red brans may serve as viable functional ingredients in extruded foods given their higher antioxidant activities. Giordano *et al.* (2017) studied the chemical composition of pigmented wheat namely yellow, purple and blue types and the distribution of the bioactive compounds in their roller-milled and pearled fractions and were compared with conventional wheat varieties that are of the red and white types. Roller-milling promoted the recovery of total dietary fiber, β-glucans, phenolic acids and anthocyanins in the bran fraction, which resulted also in a higher total antioxidant activity than the refined flour. There is a demand to find effective natural antioxidants to replace synthetic ones, which has also added to the amount of research done on cereal antioxidants such as tocopherols, tocotrienols, and phenolic acids. Tocopherols as well as other bioactive components (phytosterols,

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folates, lignans, and alkenresorcinols) have been shown to be located on the outer parts of the cereal kernel, especially the bran (Nystrom *et al.,* 2005; Mariod *et al.,* 2010). Natural antioxidants are constituents of many fruits and vegetables, and they have attracted a great deal of public and scientific attention because of their anti-carcinogenic potential and other health-promoting effects. However, consumers are quite cautious about the quality of their diet and its chemical additives to replace synthetic antioxidants with those of safer and more natural origins. This has prompted the investigation and characterization of active natural antioxidant compounds in various plant-derived foods (Zuo *et al.,* 2002). Free radical induces oxidative damage to biomolecules that cause cancer, aging, neurodegenerative diseases, atherosclerosis, malaria and several other pathological events in living organisms. Antioxidants which scavenge free radicals are known to possess an important role in preventing these free radical induced diseases. There is an increasing interest in the antioxidants effects of compounds derived from plants, which could be relevant to their nutritional incidence and their role in health. In this study, the antioxidant activity test is undertaken with determination of total antioxidant capacity (TAC) and DPPH (1, 1-diphenyl-2-picrylhydrazyl) radical scavenging assay.

2. Materials and Methods

As plant materials, mature leaves and seeds of the wheat varieties Kheri and Pavon 76 were collected from the field grown plants of the Institute of Biological Sciences, University of Rajshahi, Bangladesh. DPPH (Sigma Aldrich, India), butylated hydroxyl toluene (BHT) as standard antioxidants, sulphuric acid, ammonium molybdate, sodium phosphate, and ascorbic acid from E-Merck, Germany were used in the investigation.

2.1. Preparation of Extracts, DPPH and BHT Solution

Leaves and seeds of the plants were collected from the culture field which belongs to the Institute of Biological Sciences at the University of Rajshahi, Bangladesh in January of 2016. They were then sliced and chopped into small pieces, dried under shade, and were powdered by a hand grinder. They were weighed and placed in separate conical flasks to add solvents. Methanol and ethanol were used (200 g × 600 mL × 2 times) successively each of which took forty-eight hours on a shaker. Each of the extract filtration was done by Whatman filter paper (made in USA) at a twenty-four-hour interval in the same flask followed by evaporation until the extract was left as a scum. The extracts were then removed to glass vials, and were preserved in a refrigerator at 4°C with proper labeling. One milligram of each plant sample extracts was taken in a vial and added carefully 1 mL of solvent (methanol or ethanol) and dissolved by inverting each solution was containing 1 µg extract/1 µL. Four milligram DPPH was taken in a test tube carefully added 100 mL of solvent (methanol) and mixed then mixed gently and kept it in dark condition until uses. Then BHT 1 mg taken in a test tube in addition with 1 mL of solvent (methanol) and shacked it for dissolving. As standard antioxidant BHT was used to determine the scavenging ability which was compared with 50% DPPH and this was the IC_{50} value of the BHT for scavenging DPPH concentration.

2.2. DPPH Radical Scavenging and Measurement of the Absorbance of Solution

The DPPH scavenging activity was tested by using different concentrations of BHT and a control for comparison. Then the BHT stock solution was taken for 5, 15, 30, 60, 100, 200 and 400 µL solution and 995, 985, 970, 940, 900, 800 and 600 µL solvent (methanol or ethanol) were added in each concentration, respectively. Therefore, the concentrations of the BHT in the first seven samples were taken 5, 15, 30, 60, 100, 200 and 400 µg/µL, respectively. Finally, 3 mL of the DPPH solution were added to each of the samples to make a final volume of 4 mL for every sample. The samples were then incubated at room temperature for thirty minutes in the dark to complete the reaction. After thirty minutes of incubation absorbance of the solutions were measured at 519 nm using a spectrophotometer against blank. All the measurements were repeated three times for accuracy of the experiment, and the mean value of the absorbance was used for further calculations. A typical blank solution contained all reagents except BHT.

2.3. DPPH Scavenging Activity and IC50 Value of BHT

The percentage (%) inhibition activity was calculated from the following equation: $I = \frac{A_0 - A_1}{A_0} \times 100$; where, I is the percentage of inhibition, A_0 is the absorbance of the control and A_1 is the absorbance of the BHT.

2.4. DPPH Free Radical Scavenging Assay of Extract

For the DPPH scavenging activity test, different concentrations namely 5, 15, 30, 60, 100, 200, and 400 µg/µL solution were taken from stock solution of Kheri and Pavon 76 seed and leaf extracts of ethanol and methanol but without adding extract solution in control. Then 995, 985, 970 and 940, 900, 800, 600 µL ethanol and methanol solvents were added. Therefore, the concentration of the extract in test tubes was 5, 15, 30, 60, 100, 200, and 400 µg/µL as previously mentioned. Finally, 3 mL of DPPH solution added to each of the test tube. As a result, total volume of each of test tube was 4 mL. The test tubes were then incubated at RT (room temperature) for 30 minutes in dark to complete the reaction.

2.5. DPPH Scavenging Activity and IC50 Value of the Extract

The measurement of the absorbance, calculation of the percentage of the DPPH scavenging activity and the IC_{50} value of the extract were almost similar to the experimental procedure of BHT. Although, here, a blank solution was used and contained all reagents except the extract during the measurement of absorbance using a spectrophotometer.

2.6. Total Antioxidant Capacity Test

The total antioxidant capacity (TAC) of the samples/standard was determined by the methanol and ethanol with some modifications. Six autoclaved test tubes were needed to perform the total antioxidant capacity (TAC) test of the targeted plant extract at different concentrations. The test tubes were labeled mentioning the

concentration 1, 2, 3, 4, 5 and control. The five test tubes were respectively filled with 5 µg, 10 µg, 20 µg, 50 µg, and 80 µg/mL from the stock solution of the Kheri and Pavon 76 leaf and seed extracts. The stock solution was not added to the control test tube. The samples were mixed with 3 ml of reaction mixture containing 0.8 M sulphuric acid, 14 mM sodium phosphate and 0.4 % ammonium molybdate which were added to the test tubes. The test tubes were incubated at 95°C for ten minutes to complete the reaction. The absorbance was measured at 695 nm using a spectrophotometer against blank after being cooled at room temperature. Here, ascorbic acid was used as standard. A typical blank solution containing 3 mL of the reaction mixture, and the appropriate volume of the same solvent used for the samples/standard were incubated at 95°C for ten minutes and the absorbance was measured at 695 nm. The increased absorbance of the reaction mixture indicated an increase in the total antioxidant capacity.

2.7. Phytochemical Analysis

Phytochemical tests were carried out using various solvents while methanol, ethanol and chloroform were subjected to routine qualitative chemical analysis to identify the nature of phytochemical constituents by the standard procedures (Nithya *et al.,* 2011) as mentioned below:

Alkaloids: 1 mL of the extract was added to 2-3 drops of Mayer's reagent (dissolve 1.36 g of mercuric chloride in 60 mL of H_2O and poured onto the solution of 5 g of potassium iodide in 100 mL of H_2O). Cream color or yellow precipitation indicates the presence of alkaloids.

Terpenoids (Salkowski test): 1 mL of the extract was mixed with 2 ml of chloroform and concentrated H_2SO_4 (3 mL) was carefully added to form a layer. A reddish-brown color of the interface indicates positive results for the presence of terpenoids.

Flavonoids: In this test, 1 mL of the extract was dissolved and added in diluted NaOH + HCl. A yellow colorless solution indicates the presence of flavonoids.

Tannins: 1 mL of the extract was added to few drops of 1 % lead acetate. Yellow precipitations indicate the presence of tannins.

Phlobatanins: 1 mL of the extract was filtered and dissolved in distilled water. The filtrate was boiled with 2 % HCl solution. Red precipitations indicate the presence of phlobatanins.

Coumarins: 3 mL of 10 % NaOH was added to 2 mL of aqueous extract. The formation of the yellow color indicates the presence of coumarins.

Steroids: 1 mL of the extract was dissolved in 10 mLof chloroform, and an equal volume of concentrated H_2SO_4 was added by sides of the test tube. The upper layer turned red and the H_2SO_4 layer showed yellow with green fluorescence. This indicates the presence of steroids.

Phenols: Phenols were tested by adding 2 mL of ferric chloride solution to 2 mL of the plant extract. Appearances of a blue green color solution indicate the presence of phenols.

Saponins: Saponins were tested by boiling 5 mL of the extracts in 10 mL of distilled water in a test tube and were shaken vigorously for about thirty seconds. The test tube was allowed to settle for half an hour. The formations of froth indicate the presence of saponins.

Amino acids: 1 mL of the extract was treated with few drops of Ninhydrin reagent. The appearance of the purple color indicates the presence of amino acids.

Glycosides: The extract and alpha napthol (one ml each) were added alongside chloroform. The development of color and the results were recorded. Developments of the violet color indicate the presence of glycosides.

Carbohydrates: An alcoholic solution of substance was added to 10 % aqueous solution of alpha-naphtali. After shaking, concentrated H_2SO_4 was added along the side of the tube. The violet ring at the junction of two liquids confirms the presence of carbohydrates.

3. Results and Discussion

An antioxidant tests on the varieties of Kheri and Pavon 76 are scanty, however there are some reports on other varieties of wheat. For this reason, much attention has been focused on these two wheat varieties. These varieties are also agronomically superior in Bangladesh. Methanol and ethanol extracts were used at different concentration levels and were compared with the standard antioxidant BHT. There are some reports that explored the antioxidant effect (DPPH free radical scavenging activity) of BHT standard at seven concentration levels 5, 15, 30, 60, 100, 200 and 400 µg/mL, and was found average 24.07, 30.25, 35.87, 63.97, 88.50, 95.83 and 98.57 percentage values with BHT IC_{50} value 50 µg/mL. That means BHT is capable of scavenging the 50 % DPPH at the concentration of 50 µg/mL. Table 1 showed that the IC_{50} of the wheat varieties of Kheri and Pavon 76 leaf and seed methanol and ethanol extracts were 180, 100, 100, 190 µg/mL and 205, 55, 80, 90 µg/mL, respectively. Hence the leaf and seed methanol extract showed a higher antioxidant effect than the methanol, but this effect was not approximate to the synthetic antioxidant (BHT). The percent DPPH free radical scavenging activity of the leaf and seed methanol extract ranged from 0.51 to 66.62 %, respectively in the above-mentioned doses. On the other hand, the DPPH free radical scavenging activity of the Kheri and Pavon 76 leaf and seed ethanol extract were 2.17, 5.03, 11.74, 25.44, 39.64, 49.41, 54.34; 3.16, 16.27, 35.60, 58.58, 70.41, 77.91, 80.18 and 4.93, 10.06, 17.65, 48.82, 60.06, 64.89, 70.81; 0.39, 1.38, 17.16, 33.13, 51.68, 71.79, 77.91 %, respectively at the same afore mentioned doses (Table 1). So, it is clear that the leaf extract of Kheri and Pavon 76 of both solvents is capable of scavenging the 50 % DPPH at the dose of 180, 205 and 100, 80 µg/mL. Similarly, the seed extracts showed IC_{50} as 100, 55 and 190, 90 g/mL at the same test at diverse concentration levels. It was observed that the Kheri and Pavon 76 ethanol extract had an abstemiously high antioxidant effect although this effect was not fairly accurate to the synthetic antioxidant (BHT) (Table 1).

Table 1. DPPH free radical scavenging activity using methanol and ethanol extracts of selected seven samples and BHT with various concentrations.

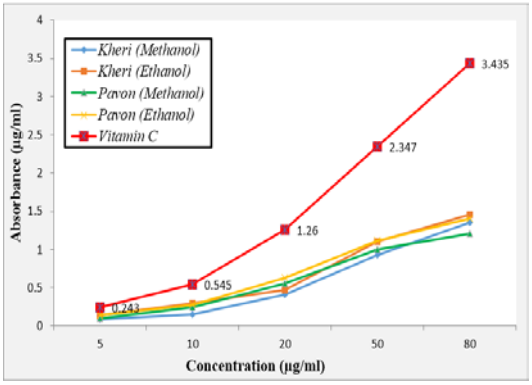
Samples		Conc. (µg/mL)	Solvents: Methanol Absorbance Mean ± SD	Methanol % of DPPH scavenging	Methanol IC_{50} (µg/mL)	Ethanol Absorbance Mean ± SD	Ethanol % of DPPH Scavenging	Ethanol IC_{50} (µg/mL)
Kheri	Leaf	5	0.688 ± 0.062	17.52	180	0.923 ± 0.11	2.17	205
		15	0.500 ± 0.308	16.88		0.896 ± 0.109	5.03	
		30	0.492 ± 0.303	18.41		0.832 ± 0.101	11.74	
		60	0.479 ± 0.295	20.84		0.703 ± 0.085	25.44	
		100	0.384 ± 0.240	38.11		0.569 ± 0.069	39.64	
		200	0.297 ± 0.183	54.60		0.477 ± 0.058	49.41	
		400	0.251 ± 0.156	62.92		0.430 ± 0.052	54.34	
	Seed	5	0.559 ± 0.032	30.18	100	0.873 ± 0.091	3.16	55
		15	0.554 ± 0.033	30.82		0.749 ± 0.089	16.27	
		30	0.503 ± 0.033	37.34		0.566 ± 0.075	35.60	
		60	0.44 ± 0.025	46.16		0.349 ± 0.066	58.58	
		100	0.417 ± 0.025	50.00		0.237 ± 0.065	70.41	
		200	0.392 ± 0.026	53.58		0.166 ± 0.066	77.91	
		400	0.340 ± 0.049	62.79		0.141 ± 0.072	80.18	
Pavon 76	Leaf	5	0.595 ± 0.205	11.25	100	0.866 ± 0.096	4.93	80
		15	0.529 ± 0.122	32.99		0.818 ± 0.091	10.06	
		30	0.473 ± 0.125	39.90		0.746 ± 0.084	17.65	
		60	0.433 ± 0.125	45.01		0.451 ± 0.059	48.82	
		100	0.389 ± 0.132	50.13		0.345 ± 0.053	60.06	
		200	0.35 ± 0.135	56.52		0.298 ± 0.051	64.89	
		400	0.287 ± 0.106	65.47		0.242 ± 0.050	70.81	
	Seed	5	0.748 ± 0.042	0.51	190	0.909 ± 0.101	0.39	90
		15	0.727 ± 0.040	3.11		0.900 ± 0.100	1.38	
		30	0.694 ± 0.039	7.68		0.756 ± 0.084	17.16	
		60	0.527 ± 0.029	29.68		0.610 ± 0.068	33.13	
		100	0.485 ± 0.026	35.30		0.441 ± 0.049	51.68	
		200	0.343 ± 0.018	54.11		0.257 ± 0.029	71.79	
		400	0.248 ± 0.013	66.62		0.202 ± 0.022	77.91	
					Control			
BHT	Conc. (µg/mL)		Absorbance Mean ± STD	% of DPPH scavenging	IC_{50} (µg/mL)			
	5		0.581 ± 0.007	24.07				
	15		0.392 ± 0.002	30.25				
	30		0.332 ± 0.002	35.87				
	60		0.281 ± 0.007	63.97	50			
	100		0.092 ± 0.002	88.50				
	200		0.356 ± 0.001	95.83				
	400		0.024 ± 0.006	98.57				

The total antioxidant effect of wheat (leaf and seed) of methanol and ethanol extracts among the five doses (5, 10, 20, 50 and 80 µg/mL) were evaluated and analyzed the absorbance of the total antioxidant activity of Kheri and Pavon 76 with the absorbance of standard vitamin C (µg/mL) (Table 2). Compared to the standard vitamin C, the methanolic and ethanolic extracts of the two varieties showed lower antioxidant activity at all concentrations. The increase in total antioxidant activity was observed with increasing the concentration of the extracts.

The results of the phytochemical activities showed that the bioactive compounds such as alkaloid, flavonoids, phenols, saponins, amino acids and carbohydrates were present in trace amount in the leaf extracts of wheat. Whereas, terpenoids, tanins, phloba tanins, steroids, phenols and glycosides, were not detected in any of the plant extracts (Table 3).

Table 2. Absorbance of wheat leaf and seed and vitamin C with various concentrations for total antioxidant activity assay.

Plant parts	Conc. (µg/ml)	Variety				Absorbance of vitamin C (µg/ml)
		Kheri		Pavon 76		
		Methanol	Ethanol	Methanol	Ethanol	
Leaf	5	0.12	0.205	0.19	0.261	0.243
	10	0.24	0.436	0.462	0.509	0.545
	20	0.62	0.712	0.874	0.900	1.26
	50	1.14	1.195	1.32	1.477	2.347
	80	1.46	1.436	1.47	1.806	3.435
Seed	5	0.091	0.136	0.098	0.133	0.243
	10	0.148	0.29	0.245	0.278	0.545
	20	0.41	0.471	0.557	0.629	1.26
	50	0.926	1.099	0.996	1.107	2.347
	80	1.346	1.454	1.2	1.403	3.435



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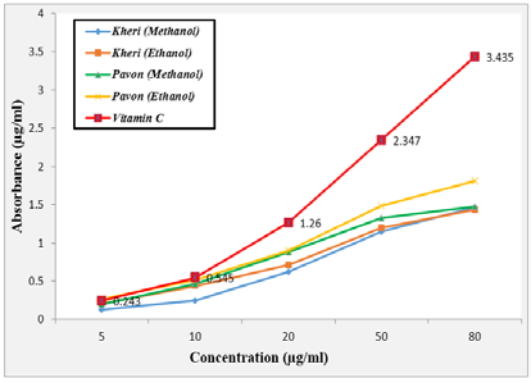

B

Figure 2. Standard absorbance of vitamin C (µg/ml) of methanol for both leaf and seed (A) and ethanol only for seed (B) extract at different concentration levels.

Table 3. Phytochemical analysis with various extracts and methods of wheat

Name of Photochemical	Test methods	Observation (colure /precipitation)	Extracts	Remarks
Alkaloids	Mayer's test	Creamy color precipitate	Methanol	+
			Ethanol	+
Terpenoids	Salkowsky test	No change is observed	Methanol	-
			Ethanol	-
Flavonoids	Alkaline reagent test	A yellow solution that turns colorless	Methanol	+
			Ethanol	+
Tannins	Lead acetate test	No change is observed	Methanol	-
			Ethanol	-
Phlobatanins	General test	No red precipitate observed	Methanol	-
			Ethanol	-
Steroids	Libermann-Burchard's test	No change is observed	Methanol	-
			Ethanol	-
Phenols	Ferric chloride test	No change is observed	Methanol	+
			Ethanol	+
Saponins	Frothing test	Formation of froth	Methanol	+
			Ethanol	+
Amino acids	Ninhydrin test	Appearance of blue color	Methanol	+
			Ethanol	+
Glycosides	General test	No change is observed	Methanol	-
			Ethanol	-
Carbohydrates	Molisch Test	Violet ring at the junction of two liquids	Methanol	+
			Ethanol	+

NB: (+) = Presence of constituents and (–) = Absence of constituents.

Antioxidant activities (by ferric reducing ability of plasma assay) of steamed bread made from whole wheat flour, partially rebranded grain flour, and refined flour were 23.5 %, 21.1 %, and 31.6 % lower, respectively, than the corresponding values of flour which suggested that the black whole wheat flour and partially rebranded grain flour are beneficial to human health (Li *et al.*, 2015). Free radicals contribute to cancer, atherosclerosis, malaria, and rheumatoid arthritis and neurodegenerative diseases (Okezie, 1999). In the scientific and medical communities, antioxidants are considered to have the ability to scavenge free radicals and reduce oxidative damage (Yu *et al.*, 2002; Mellen *et al.*, 2008). However, food processing also affects the antioxidant properties of foods to different extents. Wu *et al.* (2004) found that processing methods (including cooking and hulling) affected the oxygen radical absorbance capacity. The antioxidant activity is high compared with the effect of synthetic antioxidant (BHT) (Table 1). The free radical DPPH antioxidant activity test of wheat varieties showed that the standard BHT is capable of the scavenging of DPPH 50 % at the concentration of 50 µg/ml. The experimental result indicates that the IC_{50} value of all wheat varieties of

methanol and ethanol extracts are greater than the standard BHT. The total antioxidant capacity of wheat (leaf and seed) is higher than the standard (BHT) at five different concentrations. The antioxidant capacity of the methanolic and ethanolic extract of the wheat sample is attributed to their chemical composition and phenolic content. The present findings revealed the antioxidant activity of wheat compared with the DPPH free radical scavenging activity of BHT. The average percentages of 24.07, 30.25, 35.87, 63.68, 88.50, 95.83 and 98.57 at different concentration levels with BHT IC_{50} value of 50 µg/mL were found. That means BHT is capable of scavenging 50 % DPPH at the concentration of 50 µg/mL. Results showed that the IC_{50} value of the wheat varieties of Kheri and Pavon 76 leaf and seed are 180, 100, 100 and 190 µg/mL respectively which supports the above-mentioned findings.

The results of the phytochemical screening of wheat revealed the presence of trace amounts of alkaloid, flavonoids, phenols, saponins, amino acids and carbohydrates in all the solvent extracts (methanol and ethanol). On the other hand, terpenoids, tanins, phlobatanins, steroids, phenols and glycosides, were not detected in any of the plant extracts. The presence of these secondary metabolites in wheat confirms their antibiotic properties and usefulness for the traditional medicine practitioners to treat various ailments. Flavonoids are also known to have a wide array of therapeutic activities such as antihypertension, antirheumatism, antimicrobial, diuretic and antioxidants (Burkill, 1988; Trease and Evans, 2002). Alkaloids are formed as metabolic byproducts and have been reported to be responsible for the antibacterial activity (Doughari, 2006; Bhattacharjee and Islam, 2015). The presence of saponin enhanced the antimicrobial activity against the pathogenic microorganisms. More lipophilic flavonoids may also disrupt microbial membranes (Tsuchiya *et al.*, 1996). Phenolics and polyphenols present in the plants are known to be toxic to microorganisms (Mason and Wasserman, 1987). Phytochemicals with direct impacts on the vertebrate reproduction provide an obvious and a compelling system for studying evolutionary toxicology (Lambert and Edwards, 2017). Besides, due to the presence of antimicrobial substances they provide a source of inspiration for novel drug compounds as plant-derived medicines have made significant contribution to human health.

4. Conclusion

The studying of the antioxidant properties and bioactive compounds of the wheat varieties has justified the traditional use of plants in curing diseases. The ethanol extracts of the two tested varieties showed abstemiously-high antioxidant effects. The results revealed that the DPPH free radical scavenging assay plant extracts showed 50% scavenging ability compared with the standard antioxidant (BHT). For further investigation of safe, potent and natural source of diseases and phytochemical constituents of wheat and other plants, this technique and protocol will be helpful for researcher.

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Evaluation and Optimization of Methane Production from Different Manure Types

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Abstract

Animal manure represents an important biomass source for the production of renewable energy. In Jordan, thousands of tons of animal manure are produced annually from cows, sheep and chickens. This study aims at studying the production of methane from cow, sheep, and chicken manure to determine which type of manure gives the highest yield of methane. This study is also aimed at evaluating the effects of incubation temperature, initial pH, and trace-element supplementation (nickel, iron, zinc, copper, cobalt, manganese and boron) on the production of methane. Finally, the methane yield under optimal growth conditions will be evaluated. Methane was successfully produced from cow manure in tightly-closed stainless steel digesters that yielded the highest methane production (10 %) after thirty-five days of incubation compared to other types of manure. No methane was detected in the case of chicken manure, and only 0.1 % was detected in the case of sheep manure. The production of Methane from cow manure was then optimized using the one-factor-at-a-time method. The Methane yield increased to 47 % when incubation was done at 40°C compared to the room temperature incubation (22.9 %) and 30°C (26 %). It was also found that methanogenesis was high at acidic pH (pH 4; 42 %) when compared to other pH values (pH 5 or 6). In other experiments, the effect of trace-element supplementation was positive with the methane yield being three times greater in the presence of trace-element supplementation than that without supplementation. Finally, methane was produced under optimized conditions (40°C, pH 4, with trace-element supplementation) with the yield being higher than any other experiment (68.5%). This study highlights the feasibility of methane production from local manure types and the optimized conditions under which methane can be overproduced.

Keywords: Methane production, Optimization, Cow manure, Jordan

1. Introduction

The need for energy to assure the continuity of various human activities is obviously expanding (Owusu and Asumadu-Sarkodie, 2016). Energy can be generated from different sources that are classified into renewable and nonrenewable sources. Biomass, hydropower, wind, and solar energies are few examples of renewable energy sources, whereas petroleum, coal, and uranium are examples of the nonrenewable sources. Being a renewable energy source, Biomass is believed to have a high potential for satisfying a significant part of the global energy needs (Demirbas, 2009).

Studies have indicated that there are about 1.4 billion people in the world still lacking access to electricity especially those living in rural areas (Kaygusuz, 2012). As a result, the number of rural communities depending on energy from biomass is predicted to rise from 2.7 billion nowadays to 2.8 billion in 2030 (Owusu and Asumadu-Sarkodie, 2016; Kaygusuz, 2012). Biomass used for energy production is quite versatile and includes animal manure, municipal solid waste, waste from food processing, agricultural crops and their waste byproducts, wood and wood wastes, and aquatic plants and algae (Demirbas *et al*., 2009).

In Jordan, there are different biomass-sources that can be utilized for energy production including animal waste. It is estimated that more than 65511 tons of animal manure are produced annually from three types of animals (cows, sheep, and chicken); there are about 96100 cows, 2.5 million sheep, and 3.77 million chickens, producing about 6809, 55300, and 3402 tons of manure per year, respectively (Al-Momani and Shawaqfah, 2013). From an environmental and health point of view, this considerable amount of manure is hazardous being a major source of fresh water and groundwater pollution, nutrient leaching, ammonia emission and pathogen release if it remained untreated (Al-Momani and Shawaqfah, 2013).

Animal manure can be used for biogas production. For instance it can be produced from cows (Onwuliri *et al*., 2013) and sheep (Broucek, 2014). The resulting economic and environmental benefits of energy production from animal wastes are substantial because energy is produced, organic raw materials are converted to high-quality

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fertilizers, and the hygienic conditions are significantly improved (Sakhawat *et al*., 2013, Amankwah, 2011).

Biogas is produced by the anaerobic digestion of organic raw materials by anaerobic microorganisms such as *Bacteria* and *Archaea* (Amankwah, 2011). Biogas is usually a mixture of the gases of methane, carbon dioxide, and traces of other gases like hydrogen sulfide and hydrogen (Prabhudessia *et al*., 2014). Methane is the main component that makes biogas flammable. The concentration of methane in biogas is known to be largely dependent on several factors including the organic carbon source, the number and activity of methane-producing *Archaea* (methanogens), and the fermentation rate (Jorgensen, 2009). Methane production (methanogenesis) is also affected by several environmental conditions. In nature, methanogens favor warm temperatures as in the case of rumen's warm and constant temperature (39°C) (Madigan *et al.*, 2012). Likewise, large-scale methane production is usually run at mesophilic or thermophilic conditions (Boe and Angelidaki, 2010). The micro-organisms in the anaerobic digester are also sensitive to pH and trace-element supplementation (Mussoline *et al*., 2013; Jorgensen, 2009)

This study is aimed at producing methane from three different manure types (cows, sheep, and chicken manure) to determine which type gives the highest methane yield. This study is also aimed at evaluating the effects of different incubation conditions, namely, temperature, pH, and trace-element supplementation (nickel, iron, zinc, copper, cobalt, manganese and boron) on the production of methane using the one-factor-at-a-time method. Finally, an experiment under optimal growth conditions was carried out for the sake of this study through which the methane yield was evaluated.

2. Materials and Methods

2.1. Manure Sample Collection

Three manure types were used for methane production (chicken, cow, and sheep manure). Manure was collected from animal farms in the northern part of Jordan during January of 2015. The selected region is known to have intensive animal farming at the national level. The manure samples were collected in clean plastic bags, and were transported directly to the laboratory.

2.2. Methane Production and Evaluation

The production of methane was carried out in stainless steel digesters (20 L) according to the general procedures published in the literature (Recebli *et al*., 2015; Onwuliri *et al.,* 2013). Two kilograms of fresh manure were mixed with 4 L of water to give a ratio of 1:2 (w/v) and were transferred to the digester. The digesters were incubated at uncontrolled room temperature for twenty-one and thirty-five days without changing the initial pH and without the addition of trace elements. The digesters were tightly closed and a rubber (cascade rubber) was used to ensure an intact sealing. The digesters have two openings on the top to collect the gas and to measure the accumulated pressure. During the gas analysis time, the sampling line for gas analysis was connected with the gas analyzer (Geotechnical Instruments, England). The percent of the methane volume as well as the percent of other gases were determined at the Royal Scientific Society in Amman, Jordan**.**

2.3. Effect of Incubation Conditions on Methane Production

The effect of incubation conditions (temperature, initial pH, and trace-element supplementation) on the methane yield was studied using the one-factor-at-a-time method. In respect to the incubation temperature, there were three different constant incubation temperatures (40ºC, room temperature, and 30ºC). A closed incubator with the constant desired temperature was used and an internal thermometer was also used to monitor the temperature in the digester. After incubation, biogas was collected and analyzed as mentioned earlier (Section 2.2). The effect of initial pH on methane yield was carried out at three different initial pH values (4, 5 and 6). The manure and water mixture was adjusted to the required initial pH before starting the incubation. Regarding the effect of trace-element supplementation, six mL of mineral solution were added to ensure an additional source of iron, zinc, manganese, boron, cobalt, copper, nickel, and molybdenum (Scherer, 1983). The mineral solution was prepared by dissolving 1.5 g of $FeCl_2.4H_2O$, 70 mg of $ZnCl_2$, 100 mg of $MnCl_2.4H_2O$, 6 mg of H_3Bo_3, 190 mg of $CoCl_2.6H_2O$, 2 mg of $CuCl_2.2H_2O$, 24 mg of $NiCl_2.6H_2O$ and 36 mg of $Na_2MoO_4.2H_2O$ in 990 mL of distilled water and 10 mL of HCl (25 %) without heating. The experiment was carried out as mentioned earlier. All experiments were done in triplicates.

2.4. Methane Production under Optimized Conditions

After the determination of the individual optimal conditions (conditions that revealed the highest methane concentration) for methane production, an experiment was carried out under optimized conditions, and methane was evaluated as mentioned earlier in this study (Section 2.2).

3. Results

3.1. Methane Production from Different Manure Types

Three types of animal manure (chicken, cow, and sheep manure) were collected from animal farms in the northern part of Jordan to be used for the production of methane. After twenty-one and thirty-five days of incubation at uncontrolled room temperature, methane was produced, and its volume percent was determined. It was found that the cow manure produced the highest percent of methane volume when incubated either for twenty-one days (Table 1) or thirty-five days (Table 2) compared to other types of manure. Moreover, as expected, a higher methane content was obtained after thirty-five days (10%) (Table 2). No methane was detected from chicken manure, whereas only 0.1% was detected from the sheep manure, (Table 2). Therefore, cow manures is regarded as the most suitable manure for methane production.

Table 1. Composition of biogas produced from sheep, cow and chicken manures after 21 days. All experiments were done in triplicates.

Manure Type	CH_4	CO_2	O_2	Other gases
Sheep	0.1±1.6*	34.8±4.0	0.5±0.3	64.5±4.3
Cow	2.1±0.2	24.1±0.8	0.4±0.1	73.4±0.9
Chicken	0.0±0.0	53.1±49.9	6.5±6.6	40.4±36.5

*Mean of volume percent ± SD.

Table 2. Composition of biogas produced from sheep, cow and chicken manures after 35 days. All experiments were done in triplicates.

Manure Type	CH_4	CO_2	O_2	Other gases
Sheep	0.1±1.6*	54.2±4.7	0.3±0.2	45.3±5.0
Cow	10.0±1.9	43.2±3.5	0.2±0.1	46.1±5.5
Chicken	0.0 ±0.0	87.6±6.4	0.1±0.05	12.1±6.4

*Mean of volume percent ± SD.

3.2. Effect of Incubation Conditions on Methane Production

The Effect of incubation conditions (temperature, initial pH, and trace-element supplementation) on the methane production from cow manure was studied. Three different temperatures (room temperature, 30°C, and 40°C) were used in this study. The results showed that the highest percent of methane volume (47%) was obtained at 40°C, whereas the lowest value was obtained at the room temperature (23%) (Figure 1).

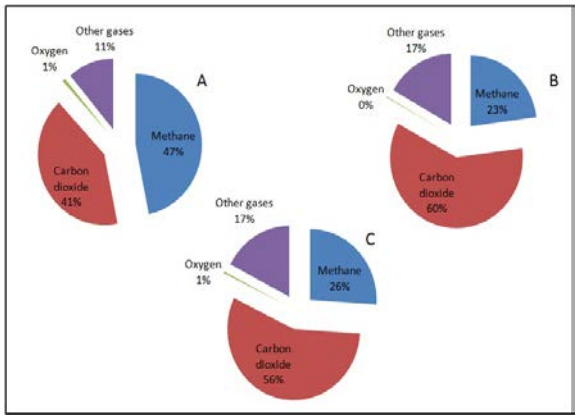

Figure 1. Composition of biogas produced from cow manure incubated at different temperatures: 40°C (A), Room temperature (B) and 30°C (C) for 35 days. The highest methane volume percent (47%) was achieved at 40°C. All experiments were done in triplicates.

Different experiments were also carried out at different initial pH values (4, 5 and 6), and the effect of initial pH on the methane production was evaluated (Figure 2). It was found that the highest volume of methane (42 %) was produced from cow manure at pH 4, and the lowest volume of methane reached (16 %) was obtained at pH 6. This indicates that the optimal initial pH value was four.

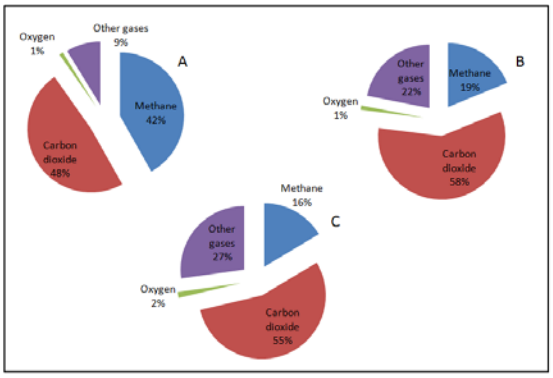

Figure 2. Composition of biogas produced from cow manure at different pH values: pH 4 (A), pH 5 (B) and pH 6 (C). All experiments were done in triplicates. Incubation was done for 35 days. The highest methane level (42%) was produced at pH 4.

Trace-element supplementation is an important factor that affects methane production. In these experiments, some elements (nickel, iron, zinc, copper, cobalt, manganese and boron) were used to support microbial growth. Figure 3 shows the enhancement of trace-element supplementation on the production of methane. The results showed that the methane production was three times higher with the use of trace elements in comparison with methane production without trace elements.

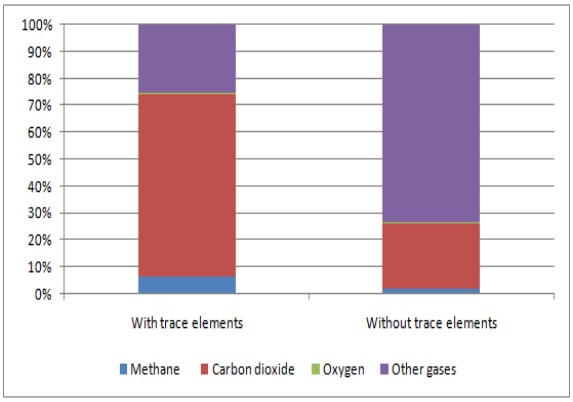

Figure 3. Composition of biogas produced from cow manure supplemented with trace element supplementation (right) and without trace-element supplementation (left). A higher percent of methane volume was obtained with trace-element supplementation. All experiments were done in triplicates. Incubation was done for 35 days.

3.3. Methane Production under Optimized Conditions

In this study, the production of methane was carried out under optimized conditions. Optimal conditions were achieved at pH4, 40°Cand addition of trace element. Results showed that the methane level was 68.5 %. This is higher than any other experiment carried out before (Figure 4).

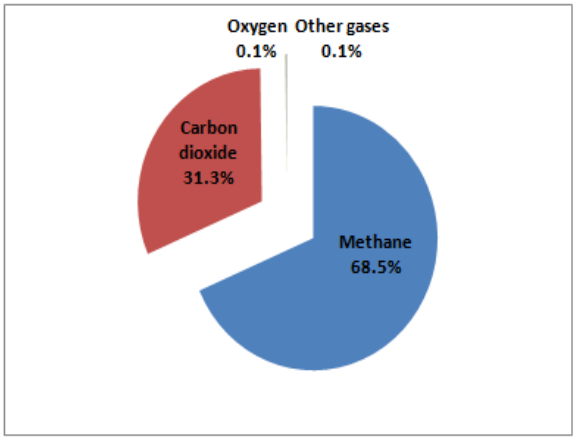

Figure 4. Composition of biogas produced from cow manure after incubation under optimized conditions (40°C, pH 4, and trace element supplementation). Incubation was done for 35 days. The methane level was the highest compared to other experiments in this study.

4. Discussion

In this study, different types of animal manure (cow, sheep, and chicken manures) were collected from animal farms in the northern part of Jordan to be used in the methane production. The selected sampling site is well-known by high livestock numbers. According to the Jordanian Ministry of Agriculture reports of 2014, there are about 3537989 sheep and 69408 cows in Jordan, and most of these animals are concentrated in the northern parts of Jordan (Jordanian Ministry of Agriculture, 2014).

According to the results of this study, cow manure as a methane bio-source gives the highest methane production, whereas chicken manure does not support methane production. This conclusion can be explained based on several factors including the number and activity of microorganisms in the manure, manure pH, and manure minerals. In more details, the cow manure may possess a higher number and activity of microorganisms in their rumen and ultimately in the manure itself than either the sheep or chicken manure. In respect to manure pH, chicken manure is more acidic than the cow manure, due to the secretion of uric acid in the chicken waste (Abu-Ashour *et al*., 2010). Accrodingly, the lower acidity in the chicken manure seems to negatively affect the number and activity of microbiota including the methanogens. Finally, in respect to the manure minerals, it was reported that the concentration of some nutrients such as (potassium and magnesium) was found to be higher in the cow manure than in the chicken manure (Abu-Ashour *et al*., 2010). Higher nutrients in the cow manure are expected to positively affect the number and activity of methanogens. The results in this study came in agreement with similar studies that reported the advantage of using cow manure as a source of methane compared to chicken manure. For instance, Recebli *et al*. (2015) reported that the biogas production from cow manure is higher than poultry manure, whereas Song *et al*. (2013) reported that the energy-production rates (kcal/h/animal) were higher when using cow manure compared to poultry manure.

In respect to the effect of incubation conditions on methane production, three incubation conditions were tested (temperature, initial pH, and trace-element supplementation). Temperature is considered as one of the important physical factors that affect methanogenesis. The literature review indicates that large scale anaerobic digesters are usually run at mesophilic conditions (the optimum growth temperature ranges between 25 to 40 °C) or thermophilic conditions (between 50 to 65°C) [Boeand Angelidaki, 2010; Mussoline *et al*., 2013]. This study showed that when temperature was increased from room temperature to 40°C, methane production was also increased. In the temperature experiments, the highest methane production was observed at 40°C (46.8%). One possible explanation of these findings is that metabolism and methanogenesis increase as temperature increases. More importantly, it must be noted that the methanogens responsible for methane liberation originate in the cow rumen which is characterized by a temperature higher than the body temperature. The rumen is usually characterized by constant and warm temperatures reaching 39°C (Madigan *et al*., 2012).

In the pH experiments, the percent of methane volume was highest at initial pH4 compared to other higher initial pH values (pH 5 and 6). Methanogenesis usually require acidic conditions to proceed (Bergey and Holt, 1994). In nature, the rumen environment is characterized by narrow pH range (5.5–7); however, this range may change depending on when the animal was last fed (Madigan *et al*., 2012). This pH range is optimal to acidogenic and acetogenic bacteria as well as methanogenic *Archaea* that which can grow and function normally at low pH range (Mussoline *et al*., 2013; Boe and Angelidaki, 2010). High pH value becomes more inhibiting to rumen bacteria and *Archaea* and sometimes toxic because ammonium is converted to ammonia at higher pH conditions (Jorgensen, 2009; Chen *et al*., 2008).

Microorganisms typically require certain nutrients for the growth and maintenance of metabolic functions. In this study, biogas production using cow manure with trace elements was compared to biogas production using cow manure without trace elements. The results showed that the manure with the additional supplementation of trace elements positively affected the methane production. Microorganisms require several trace elements for growth. Chief among these is iron (Fe), which plays a major role in the cellular respiration (Madigan *et al*., 2012). Iron is a key component of cytochromes and of iron-sulfur proteins involved in the electron transport reactions (Madigan *et al*., 2012). The cell uses magnesium (Mg) for the stabilization of ribosome, nucleic acid and membrane, and it is important for the activity of many enzymes (Madigan *et al*., 2012). Manganese (Mn) is important as an activator of many enzymes and as a component of certain superoxide dismutase. Nickel (Ni) is a cofactor in methanogens (Pramanik and Kim, 2013), whereas Cobalt (Co) is an essential trace element and an important part of the active site of vitamin B12 (Madigan *et al*., 2012). Copper (Cu) plays a role in respiration (Madigan *et al*., 2012). Our results came in agreement with Glass and Orphan (2012) who reported that methanogenic *Archaea* need a large amount of Ni, Fe and Co, and that the limited concentration of these metals can negatively affect methanogenesis. Moreover, trace- element supplementation can enhance the growth of methanogenic

Archaea, improve process stability, and increase methanogenesis (Mussoline *et al*., 2013; Kayhanian and Rich, 1995; Jorgensen, 2009).

Biogas production from animal waste can solve significant environmental microbial pollution problems by lowering the levels of contamination in the air, fresh water, and groundwater. Moreover, the utilization of the energy potential contained in the waste and the highly enriched organic fertilizer produced from this waste are important economic benefits (Girguis *et al*., 2005).

Renewable energy has become an alternative to fossil fuels with time. Generating energy from animal waste is not common in Jordan; therefore, new findings related to biogas production will increase the public awareness about the benefits and the efficiency of biogas at the national level.

5. Conclusion

Cow manure gives the highest methane production compared to chicken and sheep manure. After optimization (40°C, pH 4, with trace-element supplementation), the methane volume percent from cow manure was increased from 10 % to 68.5 %.

Acknowledgment

The authors would like to thank the Deanship of Graduate Studies at Al al-Bayt University for funding this study. Thanks are also extended to the Royal Scientific Society in Amman, Jordan for their help in the biogas analysis.

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Morphological and Molecular Identification of Fungi Isolated from Different Environmental Sources in the Northern Eastern Desert of Jordan

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Abstract

This study is aimed at isolating and identifying filamentous fungi from different environmental sources in the northern eastern Jordanian Desert. The fungal species were isolated from soil and plant parts (Fruits and leaves). The samples were collected from different geographical locations in the northern eastern Desert in Jordan. The isolation of fungi from leaves and fruits was implemented by inoculating (1ml) from serial dilutions (10^{-3}- 10^{-6}) on Potato Dextrose Agar (PDA) plates. The plates were incubated at 28˚C for one week, then the fungal colonies were observed and pure cultures were maintained. The identification of fungi at the genus level was carried out by using macroscopic and microscopic examinations depending on the colony color, shape, hyphae, conidia, conidiophores and arrangement of spores. For the molecular identification of the isolated fungi at the species level, the extracted fungal DNA was amplified by PCR using specific internal transcribed spacer primer (ITS1/ITS4). The PCR products were sequenced and compared with the other related sequences in GenBank (NCBI). Eight fungal species were identified as: *Aspergillus niger*, *Aspergillus tubingensis*, *Alternaria tenuissima*, *Alternaria alternate*, *Alternaria gaisen*, *Rhizopus stolonifer*, *Penicillium citrinum*, and *Fusarium oxysporum*. The results showed that the *Aspergillus niger* was the most abundant fungus obtained from all the locations and resources, while the *Alternaria tenuissima* was the less prevalent one. It was also noticed that two of the *Alternaria* species colonized the leaves of plants at different locations. *Rhizopus stolonifer*, *Aspergillus tubingensis*, and *Fusarium oxysporum* were isolated and identified from all resources and locations.

Keywords: Filamentous fungi, Molecular identification, Internal transcribed spacer, *Aspergillus niger* survey, Jordan

1. Introduction

A Fungus is one of the most diverse microorganisms that inhabit different environmental sources such as soil, plant parts (leaves, root and fruits), water and food sources (Maheswari and Komalavalli, 2013, Sartori *et al.*, 2013; Rebecca *et al*., 2012). The growth and distribution of fungi are affected by different environmental factors such as temperature, pH, moisture, degree of aeration, amount and type of nutrients (Gaddeyya *et al*., 2012).

Soil fungi play an important and vital role in maintaining soil fertility and productivity, and are influenced by a number of factors, including soil properties and human activities (Bao *et al*., 2012). Fungi are very important organisms that inhabit the soil. They play an important part in nutrition and processes that lead to the improvement of the health and development of the plant (Mulani and Turukmane, 2014). However, the surface of the plant leaves and fruits is inhabited by several microorganisms, including filamentous fungi and yeast that cause the deterioration and spoilage of vegetables and fruits. This makes Fungi a high-priority concern because plants are food sources for consumers and are of great economic importance to farmers (Prabakaran *et al.,* 2011). Udoh *et al*., (2015) isolated a number of fungi species from some edible fruits and vegetables that were responsible for post-harvest spoilage of some edible fruits and vegetables. To solve these serious problems, there is still a need for the isolation and identification of filamentous fungi to have that under control, and prevent their damage of the agricultural crops.

The real number of fungi is still unknown; on the other hand, only 5-13 % of the overall evaluated worldwide fungal species have been characterized (Maheswari and Komalavalli, 2013). Thus, the fungal Isolation and identification from the different environmental sources is still very essential for the viewing and recognizing of more species, editing scientific classification, evaluating their effects in nature, and supplying strains for ecological remediation, biological control, and industrial aspects (Blackwell, 2011).

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The morphology of a fungal colony in filamentous fungi results from growing as fibers (hypha), that are cylindrical, threadlike 2–10 μm in diameter structures, long up to several centimeters, with different observations of colony features such as color, size, shape visible by the naked eye which was used classically to identify fungi (Lima and Borba, 2001). The morphology of fungi was observed under a compound microscope to examine the shapes forming from the arrangement of spores (Gaddeyya *et al.,* 2012). The morphological and biochemical identifications of fungi sometimes face many problems such as: the need for a great time, requiring high skill, and generating various morpho/biotypes within one species. The use of molecular identification is fast, sufficient, reproducible, and can provide high specificity to distinguish between the species and subspecies of fungi unlike the morphological and biochemical tests used in the laboratory diagnosis of fungi (liu *et al*., 2000; Sugita and Nishikawa, 2003).

Molecular identification techniques based on total fungal DNA extraction provide a unique barcode for the determination and identification of different fungal isolates up to a species level (Landeweert *et al.,* 2003). Molecular identification using this barcode has turned into an essential tool for mycologists studying fungal taxonomy, molecular evolution, population genetics or fungus-plant interactions (Moller *et al*., 1992). The identification of fungi using molecular techniques is carried out by the sequencing of PCR amplified part of 18S rRNA genes with universal primers to fungal species (Monod *et al*., 2005; Hensel and Holden, 1996).

The northern eastern Jordanian Desert is classified as a semi-arid area based on its climatic characteristics. The climate is hot in summers, but dry and chilly in winters. The rainy season extends from November to April with the rain fall being less than 200 mL/year (Abu Sada *et al*., 2015). On other hand, agriculture is the most important economical resource in this area due to the increase in farming activities over the last two decades. This area contains a large amount of groundwater used for the irrigation of plants. According to the knowledge of the researchers, there is no study dealing with the isolation and molecular identification of fungi from the northern eastern Jordanian Desert. This study is aimed at identifying fungi isolated from the soil, leaves and fruits of vegetation that exist in this arid environment. In addition, this study looks into the geographical distribution of the isolated fungi.

2. Materials and Methods

2.1. Collection of Samples

The soil and plant part (leaves and fruits) samples were collected from the northern eastern Jordanian desert in May of 2016. The samples were separated and labeled according to their location. The soil samples were collected from the plow layer (0–15 cm in depth) of the soil at different places. About 100 g of the soil were taken and packed into labeled sterilized bottles (Gaddeyya *et al.,* 2012). The leave and fruit samples were collected by cotton swabs from the leaves and fruits of different plant families, and were placed in sterile plastic bags (Rebecca *et al*., 2012, Soni and Sharma, 2014).

2.2. Isolation of Fungi

The soil fungi were isolated by the soil dilution method. One gram of the soil sample was suspended in 10 ml of sterile distilled water to make serial dilutions (10^{-1} to 10^{-5}). One mL of each dilution was placed on Potato Dextrose Agar (PDA) containing 1 % streptomycin. The plates were incubated at 28˚C in the dark. The plates were observed for one week (Gaddeyya *et al.,* 2012; Reddy *et al.,* 2014). The leaves and fruit samples were placed and shaken in flasks filled with 100 mL of distilled water, then (0.2mL) of the sample was taken from the flasks and transferred into PDA medium with streptomycin. The cultures were incubated at room temperature in an incubator for three to five days. The fungal colonies were observed, and the pure cultures were maintained (Gaddeyya *et al.,* 2012; Javadi *et al*., 2012; Jasuja *et al*., 2013).

2.3. Macroscopic and Microscopic Examination of Isolated Fungi

The fungal morphology was studied macroscopically by observing the colony features (color, shape, size and hyphae), and microscopically by a compound microscope with a digital camera using a lactophenol cotton blue-stained slide mounted with a small portion of the mycelium (Gaddeyya *et al.,* 2012).

2.4. Molecular Identification of Fungal Species:

2.4.1. DNA Extraction and PCR Amplification

The DNA Extraction of genomic DNA from the fungi was conducted from a one-week-old PDA culture using DNeasy Plant Mini Kit (Supplied by QIAGEN). Primers (ITS1and ITS4) were used to amplify ribosomal internal transcribed spacer (ITS). PCR products were purified using the QIA quick PCR purification kit (Bao *et al*., 2012).

2.4.2. Sequencing and Analysis

The PCR products were sent for sequencing to Princess Haya Biotechnology Center, Jordan University of Science and Technology. The obtained sequences were compared with the other related sequences using BLAST search in GenBank (NCBI) (Liu *et al.,* 2000; Landeweert *et al*., 2003; Javadi *et al*., 2012).

3. Results

3.1. Macroscopic and Microscopic Features Isolated Fungi

In this study, the isolated fungi were examined on the basis of cultural, microscopic and morphological characteristics. Figure (1 – 8) show eight fungal species isolated and identified in this study

The colony morphology of *Aspergillums niger* shown in Figure 1 reveals a black color colony on the top (A1) and sulfur-yellow colonies on the reverse (A2), and the microscopic photograph shows (A3) the arrangement of conidia.

Figure 1. *Aspergillums niger* colony features on PDA (A1 Top, A2 Reverse) and conidia (A3).

Alternaria alternate features include a greenish-black surface on the top, black color on the reverse of the plate; the microscopic observed macroconidia are shown in Figure 2 (B1, B2 and B3) respectively.

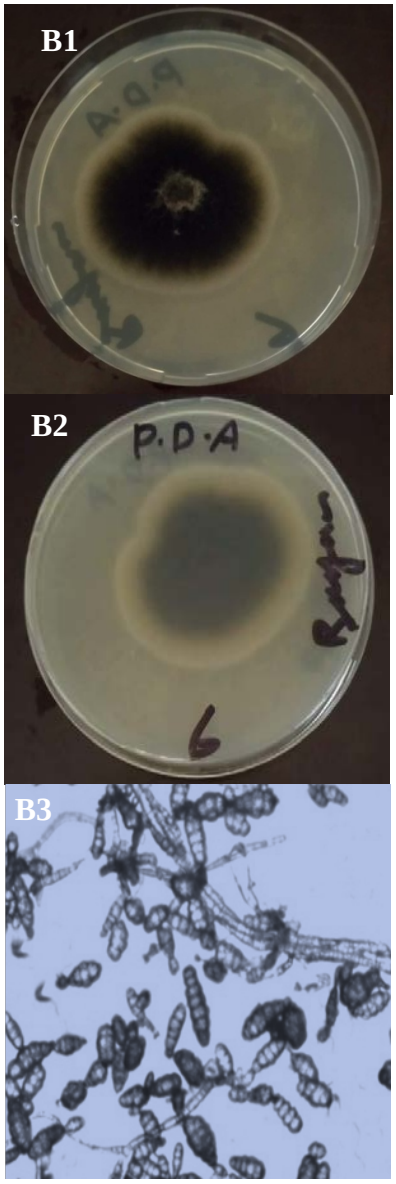

Figure 2. *Alternaria alternate* colony features on PDA (B1 Top, B2 Reverse) and chains of macroconidia (B3).

Figure 3 shows *Rhizopus stolonifer* with a deeply cottony texture of the colony having a white to gray-brown color on the top (C1), and pale white color on the reverse (C2) and dark pigment sporangium (C3).

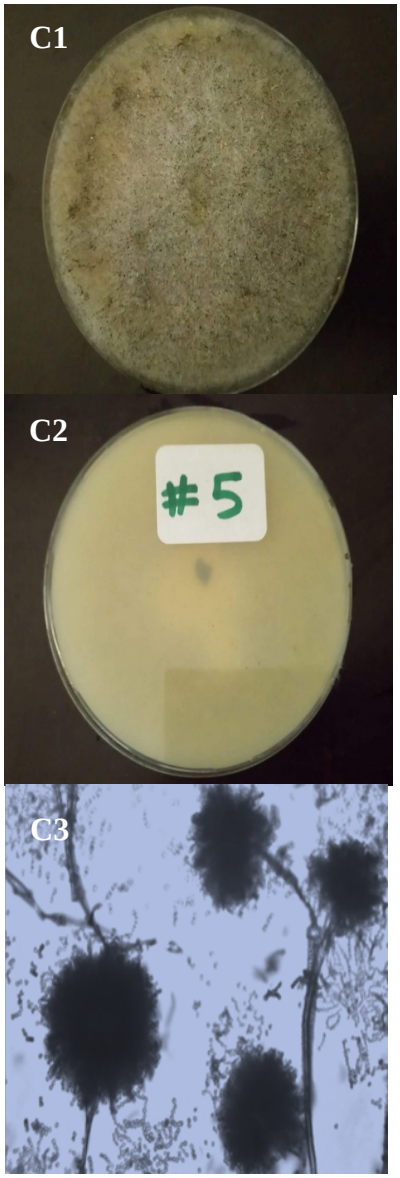

Figure 3. *Rhizopus stolonifer* colony features on PDA (C1 Top , C2 Reverse) and sporangium (C3).

The greenish-black surface on the top (D1), black color on the reverse of the plate (D2) and macroconidia of *Alternaria gaisen* (D3) as shown in Figure 4

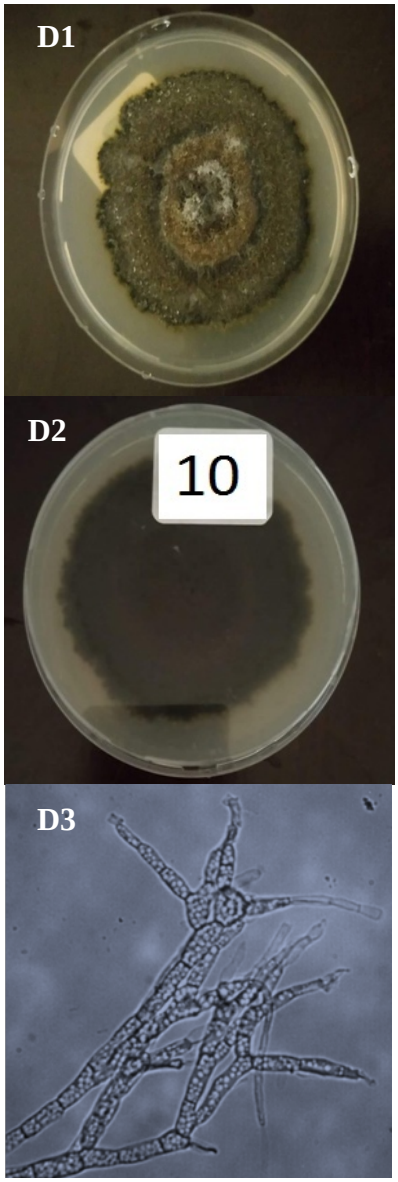

Figure 4. *Alternaria gaisen* features on PDA (D1 Top, D2 Reverse) and macroconidia (D3). .

Penicillium citrinum morphological features are shown in Figure 5 with a bluish-green surface on the top (E1), pale yellow on the reverse (E2), and the brush arrangement of phialospores (E3).

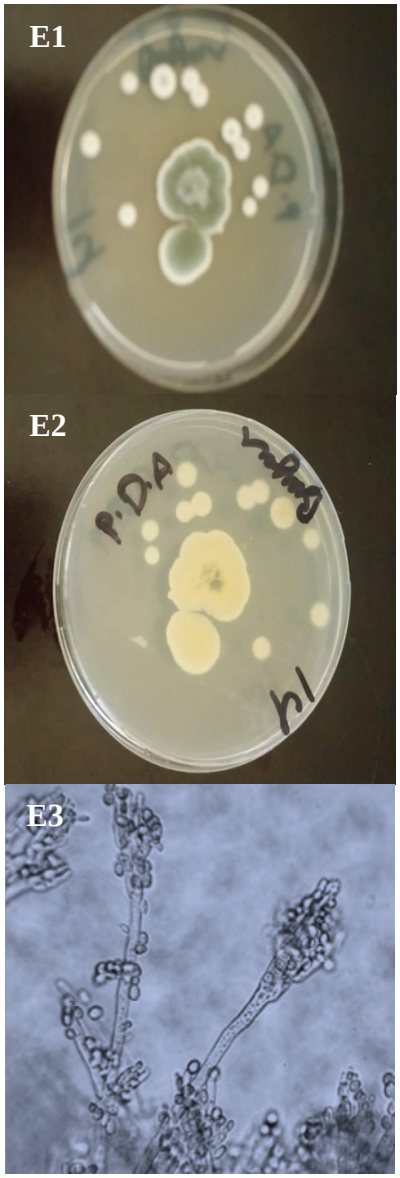

Figure 5. *Penicillium citrinum* features on PDA (E1 Top, E2 Reverse) and phialospores (E3)

The revealed morphological features of *Aspergillus tubingensis* include a white to pink color on the top (F1), light- yellow color on reverse (F2); finely wrinkled, globular, and warty conidia are shown in Figure 6.

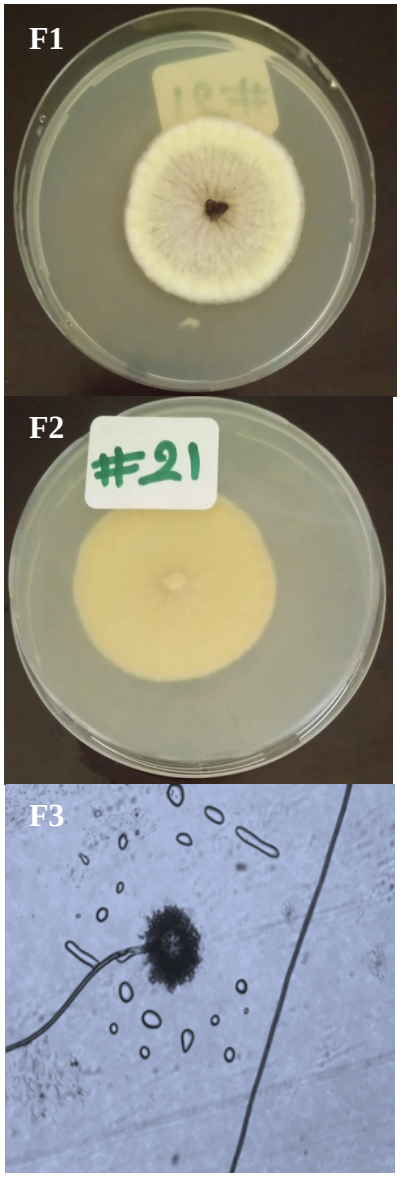

Figure 6. Plate Top, reverse and the conidia arrangement of *Aspergillus tubingensis* (F1, F2, and F3).

Figure 7 revealed the morphological characteristics of *Alternaria tenuissima* with greenish-black surface on the top (G1), black color on reverse (G2), and a microscopic photograph of the macroconidia (G3).

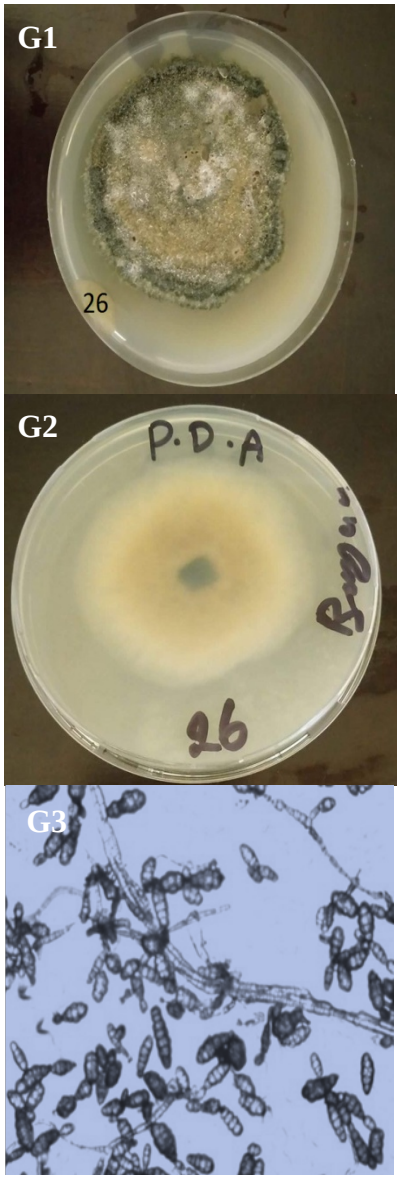

Figure 7. Plate Top (G1), reverse(G2), and the microscopic examination(G3) of *Alternaria tenuissima*.

The macroscopic identification of *Fusarium oxysporum* are shown in figure 8 (H1, H2) revealing a purple color on top, yellow color on reverse, while the microscopic photograph (H3) revealed sickle-shaped macroconidia.

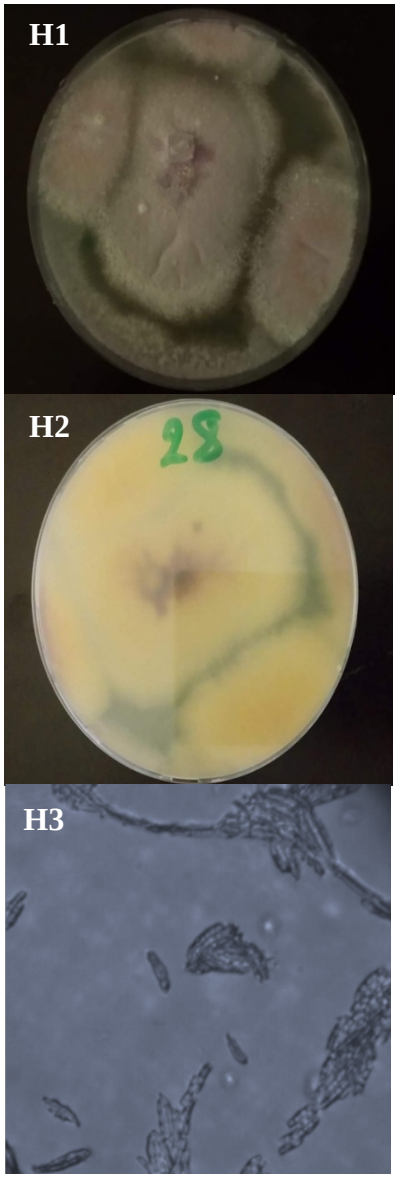

Figure 8. Plate Top, reverse and microscopic examination of *Fusarium oxysporum* (H1, H2, H3).

3.2. rDNA Sequences' Analysis

The ITS region of rDNA sequences are shown in table 1. Sequence analysis of the ITS regions of the nuclear encoded rDNA showed significant alignments of 97–99 % with the isolated fungal species.

Table 1. Identification of fungal isolates of ITS region of rRNA gene sequence.

Isolate	Species Identified	Length (bp)	Identity
BKSS1	*Aspergillus niger*	578	99%
BKSS2	*Alternaria alternate*	540	99%
BKSS3	*Rhizopus stolonifer*	731	97%
BKSS4	*Alternaria gaisen*	542	99%
BKSS5	*Penicillium citrinum*	478	97%
BKSS6	*Aspergillus tubingensis*	765	98%
BKSS8	*Fusarium oxysporum*	521	99%

3.3. Biodiversity of Fungal Isolates

The distribution of the isolated species from different sources is shown in table 2. The results showed that *P. citrinum* were isolated only from fruits, while *A. alternate* and *A. gaisen* were isolated from the leaves only, and *A. tenuissima* from the soil only, the remaining four species were isolated from all sources

Table 2. Distribution of isolated species at different sources.

Species/ Source	Fruits	Leaves	Soil
Aspergillus niger	+	+	+
Alternaria alternate	-	+	-
Rhizopus stolonifer	+	+	+
Alternaria gaisen	-	+	-
Penicillium citrinum	+	-	-
Aspergillus tubingensis	+	+	+
Alternaria tenuissima	-	-	+
Fusarium oxysporum	+	+	+

The distribution of the isolated species from different locations is shown in table 3. Eight species were isolated and identified. The result showed that *A. niger* was the most distributed species, and it was isolated from four locations, while *A. tenuissima* which was isolated from one location is the least distributed species.

Table 3. Distribution of Isolated Species at Different Locations**.**

Species/Location	Rasm Al-husan	Sabha	AL-salhiah	Al-bustaneh	Ruhbet Rakad
Aspergillus niger	+	+	-	+	+
Alternaria alternate	-	+	-	-	+
Rhizopus stolonifer	-	-	+	+	-
Alternaria gaisen	-	+	-	-	+
Penicillium citrinum	+	-	+	+	-
Aspergillus tubingensis	+	-	-	-	+
Alternaria tenuissima	-	-	-	+	-
Fusarium oxysporum	-	+	-	+	-

4. Discussion

This study was carried out to use various morphological and molecular examination methods to identify fungi isolated from the soil and plant parts (fruits, leaves) from the northern eastern Jordanian Desert. Eight fungal species were isolated and identified at the species level using rDNA ITS sequences comparison and analysis. The Isolated species belong to four classes as the following: Eurotiomycetes (*A. niger, A. tubingensis,* and *P. citrinum*), Dothideomycetes (*A. alternate, , A. gaisen,* and *A. tenuissima*), Sordariomycetes (*F. oxysporum*) and Mucoromycotina (*R. stolonifer*). Seven of these isolated fungi belong to ascomycetes, and one (*R. stolonifer*) belongs to zygomycetes. The high number and explosive dispersal of ascomycetes spore lead to a highly distributed species belonging to this phylum (Trail, 2007).

Five of the identified species (*A. niger, R. stolonifer, A. tubingensis, A. tenuissima, F. oxysporum*) were isolated from the soil. Soil is the most important source for the isolation of fungi, Chandrashekar *et al*. (2014) isolated and identified ten species belonging to three genera (*Aspergillus, Penicillium* and *Mucor)* from the rhizosphere soils in different agricultural fields of nanjangud taluk of the mysore district, karnataka, India. The results of another study conducted by Gaddeyya *et al*. (2012) revealed the isolation and identification of fifteen species belonging to six genera of fungi from the soil of agricultural fields at Salur Mandal, India. The variation and biodiversity of the isolated fungi from different geographical locations show different factors that affect the growth and distribution of fungi; these factors include soil pH, moisture content, salinity, organic carbon, nitrogen sulfur and potassium (Sharma and Raju, 2013; Yu *et al*., 2007).

Our current results showed the isolation and identification of five species from fruits (*A. niger, R. stolonifer, A. tubingensis, F. oxysporum,* and *P. citrinum*) and six species from leaves (*A. niger, A. alternate, R. stolonifer, A. gaisen, A. tubingensis,* and *F. oxysporum*) . The results came in agreement with the results of different studies that revealed the identification of the same genera

of fungi from plant fruits and leaves (Udoh *et al*., 2015, Alwakeel, 2013; Kačániová and Fikselová, 2007). On other hand, the results of the current study disagreed with the results of Bashar *et al*. (2012) that showed the isolation of nine species of fungi belonging to eight genera of filamentous fungi from the leaves and fruits of the breadfruit plant. The variations of the isolated and identified fungal species refer to the variations of plant type and environmental factors previously mentioned.

In this study the fungal isolates were firstly identified to a genus level using a morphological examination depending on the colors of colony formed at both sides, the top and reverse of the fungal cultures. The microscopic examination of the shape of the spore-producing structures was used for further identification. The morphological examination and identification of fungi are useful for the identification of isolates up to the family or genus level (Wang *et al*., 2016). However, this identification is not enough to identify the isolated fungi up to the species level (Lutzoni *et al*., 2004).

The molecular identification was carried out by DNA barcoding using the ITS region sequencing. The ITS rDNA sequences were compared to those in the databases using NCBI-BLAST. Eight species were identified using DNA barcoding with an identity range between 97 – 99 %. It is also proposed that ITS rDNA region sequence is one of the most important tools for the identification of the fungal species isolated from environmental sources (Anderson and Parkin, 2007); hence, it has been widely used to detect the soil fungal community, and as an improvement of the classical identifications. ITS rRNA genes are excellent candidates for the phylogenetic analysis because they are universally distributed, functionally constant, sufficiently conserved, and of adequate length to provide a deep view of evolutionary relationships (Madigan *et al*., 2012).

According to the results of this study, the biodiversity of the isolated fungal species is clearly observed among the geographical locations. The distribution and abundance of the fungi differ from one isolation location to another. Some species such as the *A. niger* were isolated and identified from four regions and locations, whereas *A. tenuissima* was isolated only from one region. The biodiversity of fungi refers to the physiochemical properties of the environment such as; PH of the soil, temperature, and humidity while taking into consideration that all locations belong to the desert environment.

5. Conclusion

The isolation and identification of filamentous fungi from the northern eastern Jordanian Desert displayed the presence and abundance of some economically-important fungi. As this study is the first of its kind in Jordan in that it specifically used the molecular technique which added great benefits to the process of distinguishing between similar species of fungi in comparison with the classical techniques. There is a large variation in the distribution of fungal species in different geographical locations and also from different source of isolation. Therefore, this study recommends further work to be done in the future to isolate and identify more of the filamentous fungi for taxonomy and pathogenicity investigations. It also recommends studying other types of fungi such as mycorrhiza and yeast belonging to the northern eastern Jordanian Desert.

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The Ameliorating Effect of Erythropoietin on Diabetic Neurodegeneration by Modulating the Antioxidant-Oxidant Imbalance and Apoptosis in Diabetic Male Rats

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Abstract

Diabetes mellitus (DM) is a complex syndrome which leads to multiple dysfunctions including neuropathy. Thus, the present study is aimed at assessing the effect of erythropoietin on neurodegeneration and oxidative stress in STZ diabetic rats. Group 1, control; group 2, diabetic; group 3, diabetic+erythropoietin; group 4, erythropoietin. The present results showed a significant increase in fasting blood sugar and insulin resistance while, insulin, body weight and EPO levels were decreased in the diabetic group. The protein expression of BDNF revealed a down regulation in the brain regions. Moreover, AchE was significantly decreased in the diabetic rats compared with the control ones. The oxidative status of diabetic rats was documented by an elevation in MDA and a reduction in antioxidant enzyme activities (SOD, GPx and CAT). In addition, excessive activation of PKCβ and caspase-6 were observed in the diabetic rats. This study confirms the protective effect of eerythropoietin treatment in relieving the different alterations in the diabetic rats by scavenging reactive oxygen species and modulating the PKCβ and caspase-6. EPO has been demonstrated to be a cytoprotective and block neurodegeneration by inhibiting caspase activity and apoptosis.

Keywords: Diabetes mellitus, EPO, Oxidative stress, BDNF, PKCβ and caspase-6

1. Introduction

Diabetes can lead to serious complications if it is not properly managed. Most of these complications are related to complications arising from microvascular (e.g., nephropathy, neuropathy, and retinopathy) and macrovascular (e.g., coronary artery disease, CAD; peripheral artery disease, PAD, and cerebrovascular disease) (Badran and Laher, 2012; Gupta *et al*., 2015).

It has been suggested that the term "diabetes-associated cognitive decline" (DACD) describes a state of mild to moderate cognitive impairment, in particular psychomotor slowing and reduced mental flexibility, not attributable to other causes. In addition, it is now clear that diabetes increases the risk of Alzheimer's disease (AD), vascular dementia and any other type of dementia (Chawala *et al*., 2016).

Erythropoietin (EPO), a glycoprotein with a molecular weight of 34 kDa, was identified as a cytokine responsible for the production of erythrocytes (Broxmeyer, 2013; Palis, 2014). EPO has various effects such as modulation of inflammation, slowing down of apoptosis, stimulation of angiogenesis and limitation of reactive oxygen species production. Therefore, EPO can protect neurons with a combination of these effects (Genc *et al*., 2004). Previous studies have reported that there may be an association between EPO levels and hypoglycemia, which suggests a potential protective effect of EPO in the treatment of diabetes (Choi *et al*., 2011; Zhang *et al*., 2014; Chen *et al*., 2015).

The present study is aimed to assess the effect of erythropoietin on neurodegeneration and oxidative stress in STZ-induced diabetes in male rats.

2. Materials and Methods

2.1. Chemicals

Streptozotocin (STZ) and Erythropoietin (Epo) were purchased from Sigma–Aldrich Chemical Company (St. Louis, MO, USA).

2.2. Animals

Male *Wistar* rats (200-250 g) were obtained from the Faculty of Medicine of Alexandria University in Egypt. They were housed under controlled conditions (25±1°C constant temperature, 55% relative humidity, 12 h lighting cycle) for two weeks prior to the experiment for acclimation and received standard diet and water *ad libitum.* All animal procedures and the experimental protocols were carried out according to the guidelines of the National Institutes of Health (NIH).

2.3. Experimental Design

Forty male Wistar rats were randomly divided as the following:

Control group: The rats of this group were treated intraperitoneally citrate buffer (pH 4.5).

Diabetic group: The rats of this group were treated intraperitoneally with a single injection of streptozotocin (STZ) at a dose of 50 mg/kg body weight dissolved in citrate buffer (Hongying *et al*., 2013).

Diabetic and erythropoietin treated group: Diabetic rats were treated intraperitoneally with erythropoietin injection at a dose of 40 µg/kg body weight three times per week for five weeks after diabetes induction (Bianchi *et al*., 2003).

Erythropoietin treated group: The rats of this group were treated intraperitoneally with erythropoietin only at a dose of 40 µg/kg body weight three times per week for five weeks

The body weight records were measured at the end of the experiment. At the end of the experimental period all rats were anesthetized with ether. Blood samples were collected by cardiopuncture and plasma samples were separated.

2.4. Induction of Diabetes

Diabetes was induced chemically by intraperitoneal injection of streptozotocin (STZ) at a dose of 50 mg/kg body weight (Hongying *et al.,* 2013). The fasting blood glucose level was measured 72 h after the STZ treatment, using reagent strips (Accu-ChekW, Roche) with a drop of blood obtained by tail-vein puncture. Animals were considered diabetic if blood glucose values were higher than 200 mg/dl.

2.5. Preparation of Serum

The heparinized blood samples were centrifuged at 3000 xg for fifteen min. Serum was separated and then stored at -20 °C until the biochemical analysis.

2.6. Preparation of Brain Homogenate

Whole tissues of the brain were obtained by dissection, cleaned from the adhering matters, and washed with physiological saline. Then the brain tissues from each rat was minced and homogenized in phosphate buffer (pH 7.4). Homogenates were centrifuged at 10000 xg for twenty minutes at 4°C and the clear supernatants were separated for lipid peroxidation and antioxidant enzyme determination.

2.7. The Biochemical Parameters

Fasting blood sugar and insulin levels were determined according to the methods of Bergmeyer and Berndt (1974) and Valverde *et al*. (1988), respectively. The insulin resistance was estimated by Matthews *et al*. (1985) HOMA score = fasting insulin (µIU/L) x fasting glucose (mmol/L)/22.5. Sandwich enzyme-linked Immune-Sorbent assay technology was used for erythropoietin (EPO) determination (Haroon *et al*., 2003). The brain derived neurotrophic factor (BDNF) activity was determined by using sandwich enzyme immunoassay (EIA) (Elfving *et al*., 2010). Determination of acetylcholinesterase (AchE) activity (EC 3.1.1.7) was assayed according to Srikumar *et al*. (2004). Malondialdehyde (MDA) (EC 1.2.1.3) (Ohkawa *et al*., 1979), superoxide dismutase (SOD) (EC 1.15.1.1) (Nishikimi *et al*., 1972), glutathione peroxidase (GPx) (EC 1.11.1.9) (Paglia and Valentine, 1967), catalase (CAT) (EC 1.11.1.6) (Aebi, 1984) were estimated. Protein kinase c (PKC) (EC 2.7.11.13) was measured by the method of Keranen and Dutil (1995). Caspase 6 was assayed by using commercial kits (www. MyBioSource.com) (Cat. No. MBS721980).

2.8. Statistical Analysis

Statistical analyses were performed using the SPSS package for Windows version 22.0. Data were expressed as mean±SE. One-way ANOVA was used to analyze differences among groups. Differences among groups were considered statistically significant at $P \leq 0.05$.

3. Results

3.1. The Effect of Erythropoietin (EPO) on Fasting Blood Sugar, Insulin and Insulin Resistance Levels in the Different Studied Groups:

Table 1 illustrates that the fasting blood sugar levels and insulin resistance were significantly ($P \leq 0.05$) increased in the diabetic group compared to the control group. In contrast, insulin levels and body weight were decreased in the diabetic group. Meanwhile, the treatment of diabetic rats with EPO caused a significant ($P \leq 0.05$) decrease in the fasting blood sugar levels and insulin resistance and an increase in insulin levels and body weight compared to the untreated diabetic group. In the EPO group, the fasting blood sugar and insulin levels were more or less similar to the control values.

Table 1. The effect of erythropoietin (EPO) on fasting blood sugar, insulin levels, insulin resistance and body weight in the different studied groups.

Parameters	Experimental groups			
	Control	Diabetic	Diabetic+ EPO	EPO
Fasting blood sugar (mg/dL)	96.333[a] ±2.422	300.167[b] ±32.307	176.429[c] ±8.772	93.833[a] ±3.251
Insulin level (µlU/mL)	5.794[a] ±0.046	3.577[b] ±0.368	5.113[a] ±0.251	5.650[a] ±0.143
Insulin resistance	26.954[a] ±2.997	47.953[b] ±3.894	38.338[c] ±2.631	23.416[d] ±2.773
Body weight (g)	225[a] ±11.452	178.367[b] ±12.684	190.252[c] ±12.541	220.250[a] ±13.351

Values are expressed as means±S.E; n=10.

Mean values within a row not sharing a common superscript letter (a, b, c) were significantly different ($P \leq 0.05$)

Table 2. EPO, BDNF and AchE levels in the different studied groups.

Parameters	Experimental groups			
	Control	Diabetic	Diabetic+EPO	EPO
EPO level (pg/ml)	112.974[a] ±1.803	98.581[b] ±1.292	114.806[a] ±1.928	110.769[a] ±3.724
BDNF level (ng/ml)	0.018[a] ±0.001	0.013[b] ±0.001	0.015[c] ±0.002	0.019[a] ±0.001
AchE level (ng/ml)	0.869[a] ±0.063	0.529[b] ±0.061	0.659[c] ±0.097	0.846[a] ±0.066

Values are expressed as means ± S.E; n=10.

Mean values within a row not sharing a common superscript letter (a, b) were significantly different ($P \leq 0.05$)

3.2. The Effect of EPO on MDA, SOD, GPx and CAT Levels in the Different Studied Groups:

Table 3 shows that MDA was significantly ($P \leq 0.05$) increased in the diabetic group compared to the control ones. On the other hand, the levels of antioxidant enzyme activities (SOD, GPx and CAT) were significantly ($P \leq 0.05$) decreased in the diabetic group compared to the control. EPO administration significantly improved the MDA, SOD, GPx and CAT levels in comparison with the untreated diabetic rats.

Table 3. The effect of EPO on MDA, SOD, GPx and CAT levels in the different studied groups.

Parameters	Experimental groups			
	Control	Diabetic	Diabetic+EPO	EPO
MDA (nmol/mL)	7.98[a] 0.91	38.16[b] 0.68	14.6[c] 1.14	7.90[a] 0.53
SOD (U/mL)	75.40[a] ±4.547	26.250[b] ±4.678	46.95[c] ±4.883	73.90[a] ±6.656
GPx (nmol/min/mL)	64.30[a] ±6.165	21.85[b] ±2.943	45.55[c] ±3.940	64.75[a] ±5.220
CAT (nmol/min/mL)	46.650[a] ±4.626	16.60[b] ±2.583	31.05[c] ±3.268	45.70[a] ±4.497

Values are expressed as means ± S.E; n=10.

Mean values within a row not sharing a common superscript letter (a, b, c) were significantly different ($P \leq 0.05$)

3.3. The Effect of EPO on PKC and Caspase 6 in the Different Studied Groups.

Table 4 shows that PKC and Caspase 6 levels were significantly ($P \leq 0.05$) enhanced in the diabetic group compared to the control group. The treatment of diabetic group with EPO caused a significant ($P \leq 0.05$) decrease in the PKC and Caspase 6 levels compared to the untreated diabetic group.

Table 4. The effect of EPO on PKC and Caspase 6 in the different studied groups**.**

Parameters	Experimental groups			
	Control	Diabetic	Diabetic+EPO	EPO
PKC (ng/mL)	6.94[a] ±0.54	32.84[b] ±2.45	12.8[c] ±0.91	6.12[a] ±0.45
Caspase 6 (ng/mL)	20.0[a] ±1.58	59.8[b] ±1.92	36.2[c] ±2.39	20.8[a] ±1.79

Values are expressed as means ± S.E; n=10.

Mean values within a row not sharing a common superscript letter (a, b, c) were significantly different ($P \leq 0.05$)

4. Discussion

Type 2 diabetes (T2D) is one of the modifiable risk factors for Alzheimer's disease (AD) and has a 1.5 to 2 fold increased risk to develop cognitive impairment (Yael *et al*., 2010). AD is characterized by a progressive decline in the cognitive function (Revett *et al*., 2013; Honjo *et al*., 2012). Insulin plays a major role in the memory and brain function in patients with AD by regulating neuronal functions, neurogenesis and neurotransmitter regulation (Myint *et al*., 2013).

The present hyperglycemia status was attributed to STZ-induced depletion in the secretion of insulin by partial destroying pancreatic β-cells, reduction in insulin production, resulting in enhancement of glucose level that in turn causes protein glycosylation (Forde and Medeiros, 2008) and poor glucose utilization by tissues (Eliza *et al.*, 2009). These results came in accordance with Selvan *et al.* (2008) and Dewanjee *et al.* (2009). This glucose overloading can activate many metabolic or signaling pathways that not only attempt to dispose excessive glucose, but also generate more reactive oxygen species, leading to oxidative stress and β-cell failure together with the evidence of a low-level antioxidant capacity in β-cells which are thought to be responsible for secondary diabetic β cell failure (Wu and Yan, 2015). Similar results were reported by another researcher who found that the fasting blood glucose level in diabetic rats was significantly higher than that in the control rats in the first week (Hwang *et al.*, 2017). Hyperglycemia is also associated with the consequences of hyperinsulinemia, insulin resistance and glucose intolerance (Nasry *et al.*, 2013). EPO is under investigation for the treatment of a variety of diseases, and appears to be especially suited for the treatment of disorders of metabolism including diabetes mellitus (DM). In addition to EPO utility for the treatment of anemia, EPO can improve cardiac function, reduce fatigue, improve cognition in patients with DM, and regulate cellular energy metabolism, obesity, tissue repair and regeneration, apoptosis, and autophagy in experimental models of DM (Maiese, 2015). Therapeutic strategies that can specifically target and control EPO and its signaling pathways hold great promise for the development of new and effective clinical treatments for DM and the complications of this disorder.

However, EPO supplementation significantly lowered the fasting blood glucose compared with the untreated diabetic group. Katz *et al.* (2005) reported that after one

week of treatment, all EPO-treated mice had lower blood glucose levels which were further reduced during the second week of treatment.

Regarding the possible mechanism(s) for EPO-induced attenuation of hyperglycemia, it was found that EPO increases the glucose utilization that seems to be responsible for the reduction of hyperglycemia in the diabetic rats (Caillaud *et al*., 2015). Moreover, a pleiotropic action of EPO is not associated with endogenous insulin, indicating an insulinotropic action of EPO. EPO has been demonstrated to show antioxidant-like activity. Also, reduction of hyperglycemia induced by a bolus injection of EPO seems to be related to the antioxidant-like action. In addition, EPO enhanced the insulin sensitivity in diabetic rats. This result is reasonable because EPO has been linked to many signals that occur after activation of receptors (Si *et al*., 2013).

Several previous studies have demonstrated that treatment with EPO improves insulin sensitivity and reduces insulin resistance in dialysis patients, obesity and metabolic syndrome mice and 3T3L1 adipocytes, via EPO receptors in insulin-responsive tissues, including the muscle and liver, or indirectly through the correction of anemia (Pan *et al*., 2013; Zhang *et al*., 2014). Chen *et al.* (2015) demonstrated that treatment with EPO reduced fasting blood glucose (FBG), ameliorated glucose tolerance and insulin sensitivity, and lowered glyconeogenesis in experimental diabetic rats. A previous randomized placebo controlled trial (Benstoem *et al*., 2015) demonstrated that insulin sensitivity is improved following treatment with EPO in healthy humans. He *et al.* (2010) suggested that EPO can protect neonatal islet cells in the porcine model by upregulating β-cell lymphoma-2 and down-regulating β-cell-associated X protein and caspase 3.

The body weight decline in diabetic rats may be attributed to the association of diabetes with increased glycogenolysis, lipolysis, and gluconeogenesis. These biochemical activities result in muscle wasting and loss of tissue protein (Ewenighi, 2015). Treatment of diabetic rats with rHuEPO produced significant increase in both body weight and food intake (Khalaf *et al*., 2015).

Brain derived neurotrophic factor BDNF is an important neurotrophic factor in the nervous system that can promote nerve regeneration and maintain normal neuronal function. BDNF may play a role through binding with receptor P75 and TrkB on nerve cells and activating various signaling pathways (Bathina and Undurti, 2015). The protein expression studies of BDNF revealed a down regulation in the brain regions of the diabetic rats. The results of the present study were in agreement with the report of Yang and Gao (2017) who showed a decrease in the expression of this neurotrophin in diabetes.

The increased oxidative stress which occurred as a result the pre-existing diabetes in the rats could cause a reduction in the expression BDNF, which could have contributed to the memory impairments observed in the diabetic rats. A positive correlation between brain BDNF concentration and cognitive performance has been described, while decreasing BDNF production has been proposed as one possible pathogenetic factor for Alzheimer's disease and major depression (Zoladz and Pilc, 2010). Interestingly, BDNF levels are decreased in patients with diabetes mellitus type 2 (DM2), and have been inversely correlated with plasma glucose and insulin resistance as assessed by a homeostatic model assessment (Ortíz *et al*., 2016).

EPO has been shown to protect primary hippocampal neurons by increasing the expression of brain-derived neurotrophic factor (Viviani *et al.,* 2005). Wang and Xia (2015) demonstrated that EPO can upregulate BDNF expression in the diabetic rats. Promising research is emerging around–BDNF, in cognitive dysfunction associated neurological disorders. In the brain, BDNF promotes the optimum communication between neurons by enhancing "plasticity" at the synapse. BDNF plays an important role in the survival of neurons, their growth (axons and dendrites), and the formation and function of the synapse (Blurton-Jonesa *et al.,* 2009). Without sufficient BDNF and other neurotrophic factors, neurons die. Cholinergic transmission in the brain cortical and hippocampal regions plays a fundamental role in memory (Zanardini *et al*., 2016).

Under normal conditions, AChE activity is a specific marker of cholinergic neurons in the cerebral cortex and hippocampus. It plays a critical role in the modulation of the cholinergic pathway (Mao *et al*., 2014). The reduction in AchE in the diabetic group may be attributed to the insufficient activity of AChE as a contributing factor in the development of diabetic neuropathy (Baquer *et al.,* 2011). AChE is one of the important membrane bound enzymes in the brain that influence the acetylcholine levels. Several earlier studies reported a decrease in the AChE level in diabetic male rats with a significant increase in lipid peroxidation in the brain tissue (Kumar *et al.*, 2015).

Elevated MDA is regarded as a specific indicator of lipid peroxidation during oxidative impairment. In addition, oxidative injury could also destroy the antioxidant defense system, such SOD, GPx and CAT. In fact, it was previously found that oxidative brain damage caused by oxidative stress contributed to the serious impairment of learning and memory deficits during aging in rats (Wu *et al*., 2013; Mao *et al.*, 2014).

It is well documented that MDA is a stable end product of free radicals induced by lipid peroxidation. Thus, MDA serves as a reliable marker for the assessment of free radical induced damage to tissues. In diabetic patients, a major factor that is responsible for enhanced free radical generation is hyperglycemia through auto-oxidation of glucose (Kangralkar *et al*., 2010) protein glycation, lipid peroxidation, and low activities of antioxidant enzymes. The mechanism of the enhancing of the oxidative stress might be due to protein glycation and inhibition of antioxidant enzyme activities (SOD, GPx) (Sheweita *et al*., 2015). In addition, oxidative injury could also destroy the anti-oxidant defense system, such SOD, GPx and CAT.

On the other hand, erythropoietin has an antioxidative effect as indicated by decreasing the level of MDA, resulting in decreasing the amount of oxidative stress and subsequent lipid peroxidation (Ahmadias *et al.,* 2013). In the Chen *et al*. (2015) study, administration of EPO decreased the level of MDA, and increased the activity of antioxidant enzymes, including SOD and GPx, compared with the diabetic model group. EPO increases antioxidant enzyme activities by increasing levels of nuclear factor erythroid 2-related factor 2 (Nrf-2) to the nucleus where it

binds and activates the antioxidant response element (Jin *et al.*, 2014; Genc *et al.*, 2010). EPO also causes the increased activity of the antioxidant protein glutathione peroxidase *in vivo* (Kumral *et al*., 2005), superoxide dismutase (Barichello *et al*., 2014).

Increased activation of PKC isozymes has been observed in cancer (Toton *et al*., 2011), diabetes, ischemic heart disease, acute and chronic heart disease (Palaniyandi *et al.*, 2009) and in a number of neurological diseases, including stroke, Parkinson's disease (Burguillos *et al.*, 2011), dementia, Alzheimer's disease, pain and even in psychiatric diseases, including bipolar disease (Davari *et al*., 2013).

Preclinical research suggests that hyperglycemia leads to the activation of PKCβ, which may play an important role in mediating the microvascular disease complications of retinopathy, nephropathy, and neuropathy. Hyperglycemia leads to the chronic activation of PKCβ, causing aberrant signaling and a variety of pathologies such as cytokine activation and inhibition, vascular alterations, cell cycle and transcriptional factor mis-regulation and abnormal angiogenesis (Geraldes and King, 2010).

The present study showed a remarkable elevation in caspase-6 activity in the diabetic rats. Some data have suggested that elevated glucose level may increase caspase-6 expression levels and induce oxidative stress, leading to apoptosis (Maedler *et al*., 2009). Similarly, caspase activation has been noted before the development of neurofibrillary tangles of Tau in the brain of tau transgenic mice (De Calignon *et al*., 2010). Caspase-6 activation is one of the main characteristics of many neurodegenerative diseases, including diabetes-associated cognitive deficits and AD (O'Brien and Wong 2011; Zawada *et al.*, 2015 and Sadeghi *et al*., 2016).

Wang *et al.* (2017) reported that EPO treatment significantly decreased cell apoptosis. This result may be associated with an EPO-induced decrease in the levels of caspase-6 and oxidative stress. EPO also blocks Apaf-1 activation, and prevents the early activation of several caspases (Park *et al*., 2011; Shang *et al*., 2012).

Neuroprotective and neurotrophic effects of EPO have been shown in different experimental brain damages. Kumar *et al.* (2010) proposed that the beneficial effect of EPO mediated in STZ dementia may be attributed to its multiple effects, including antioxidative (Wang *et al*, 2009), anti-inflammatory and neuroprotective actions (Granic *et al.*, 2009; Salminen *et al*., 2009).

EPO was effective in preventing neuronal apoptosis in many types of neurodegenerative conditions in the brain and spinal cord (Maiese *et al.*, 2012). Treatment with EPO decreases cellular damage caused by ROS/RNS, including lipid peroxidation, protein carbonylation (Barichello *et al*., 2014), and protein nitrosylation (Lu *et al*., 2012), thus, preventing downstream damaging effects on cells that lead to apoptosis.

5. Conclusion

Hence, it may be concluded that EPO has shown ameliorative effects on neurodegenerative and brain oxidative stress. Further studies should be directed towards knowing new biomarkers for AD, enabling improved understanding, prediction and prevention of this major clinical problem of diabetes complications

Conflict of interest

The authors declare that they have no conflict of interest.

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