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Jordan Journal of Biological Sciences (JJBS) aims to publish high quality manuscripts and provide readers worldwide with high quality peer-reviewed scholarly articles on a wide variety of biological sciences such as Cell biology, developmental biology, structural biology, microbiology, entomology, toxicology, molecular biology & genetics, biochemistry, biotechnology, biodiversity, ecology, marine biology, plant biology, animal biology, physiology, and bioinformatics. JJBS is a refereed, peer reviewed quarterly international journal financed by the Scientific Research Support Fund, Ministry of Higher Education and Scientific Research in cooperation with the Hashemite University, Jordan. JJBS has been indexed by SCOPUS; CABI's Full-Text Repository, EBSCO, Science Citation Index- Zoological Abstract and recently has been included in the UGC India approved journals. JJBS is currently under evaluation to be indexed in Thomson Reuters, National Library of Medicine's MEDLINE\ Pub Med system and others.

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 would like to express my deep sorrow and sincere condolences to the Scientific Community in Jordan and abroad, as well as to the family and friends of Professor Dr. Hakam F. Al-Hadidi, Editorial Board Member, who lately passed away. On this occasion, we in the Editorial Board remember the tremendous efforts exerted by him to improve the Journal. Even if Prof. Hadidi has left our life, I am sure that he will always continue to be present in our hearts and minds as a close friend and respective colleague. May God's endless mercy be upon his soul and paradise be his last residency.

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.

In the coming year, it is my vision to have JJBS publish a combination of manuscripts documenting rigorous studies in the area of biological sciences, and one or more manuscripts from distinguished scholar on recent advances in molecular biology. As you read throughout this volume of JJBS, I would like to remind you that the success of our journal depends directly on the number of quality articles submitted for review. Accordingly, I would like to request your participation and colleagues by submitting quality manuscripts for review. One of the great benefits we can provide to our prospective authors, regardless of acceptance of their manuscripts or not, is the feedback of our review process. JJBS provides authors with high quality, helpful reviews to improve their manuscripts.

Moreover, and as always, my thanks are also extended to the Hashemite University and Jordanian Scientific Research Support Fund for their continuous financial and administrative support to JJBS. I would like to highlight and proudly thank the group of authoritative reviewers, both local and international, who have done an outstanding work. We are honored to have you on our review list and many thanks for your valuable mentorship and contributions you provided to authors. Indeed, we count on your excellent reviews to include only high quality articles worthy of publication in JJBS. Together, we strive to make JJBS reach a remarkable rank among other international journals. I very much appreciate your support to make JJBS one of the most authoritative journals in biological sciences.

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Prof. Khaled H. Abu-Elteen
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Harvesting of *Scenedesmus obliquus* by Bioflocculation: Appropriate Chitosan Concentrations with Various pH Values at Different Growth Stages

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Abstract

The current study evaluates harvesting microalga *Scenedesmus obliquus* at different growth phases using chitosan, an anionic bioflocculating polymer. Different concentrations of chitosan were applied at different cultural pH levels and at different growth stages. The flocculation efficiency was evaluated in *S. obliquus* cultures with various cell densities. The optimum chitosan level for flocculating *S. obliquus* varied according to the cultural growth phase, cultural pH, and cells density. The highest flocculation efficacies (89.07 % and 88.48 %) at the late log phase were obtained when 80 ppm and 10 ppm of chitosan were applied to cultures with pH 10 ($OD_{680nm} \approx 1$) and pH 7 ($OD_{680nm} \approx 3$), respectively. Moreover, during the early stationary phase, the maximum coagulation value was achieved when 40 ppm of chitosan was used for cultures with pH 9 and $OD_{680nm} \approx 3$. At the late stationary growth phase, the highest flocculation efficacy (81.68% and 81.19 %) was achieved in cultures adjusted to pH 6 and treated with 20 ppm of chitosan and in the culture with pH 9 treated with 60 ppm of chitosan, correspondingly. Flocculation efficiency of *S. obliquus* could be improved by selecting a proper chitosan concentration according to the culture conditions (growth phase, pH, and cells density).

Keywords: *S. obliquus,* Bioflocculation, Chitosan, Growth stages, culture densities

1. Introduction

Microalgal biomass is considered as a sustainable feedstock for biofuel production, feed staff, nutraceutical and pharmaceutical products (El-Baz *et al.,* 2016; Hoh *et al.,* 2016; Lal and Das, 2016; Eida *et al.,* 2018). However, the energy richness of algal biomass, its diluted growth being around 0.02–0.05 % dry solids (Zamalloa *et al.,* 2011) and the small cell size being mostly below 30 µm (Grima *et al.,* 2003) represent the biggest challenges for microalgae harvesting, its commercial production, utilization and application.

The negative charge of the algal cells surface, that makes it well dispersed in suspension growth, in addition to the low density of algal cells in growth medium are considered among the imperative barriers standing in the way of expanding and scaling up microalgae production (Reynolds, 1984; Edzwald, 1993; Milledge and Heaven, 2013; Xia *et al.,* 2017). Such harvesting difficulties make the discovery and adoption of a harvesting technique that is efficient, cost-effective and environment-friendly extremely problematic for commercializing the microalgae production especially in biofuel production which requires a mass production of algal cells (Ghernaout and Ghernaout, 2012; Milledge and Heaven, 2013; Alam *et al.,* 2016).

Several methods can be implemented for harvesting microalgae from their cultures. These techniques include centrifugation, filtration, flocculation, flotation and sedimentation or a combination of two or more of these methods; although there is no common scheme for harvesting all microalgal species yet (Mata *et al.,* 2010; Milledge and Heaven, 2013; Shen *et al.,* 2009).

Flocculation is one of the most commonly applied techniques. It takes advantage of flocculant positive charge to form flocs with the negatively charged cells and improve its settling rate (Xu *et al.,* 2013; Matter *et al.,* 2018). Different inorganic chemical materials (including aluminum and ferric based salts) have been recognized for the coagulation of microalgae. In spite of the prevalence and the high efficiency of these methods, they have several possible environmental disadvantages (Fast and Gude, 2015). Thus, flocculation using natural polymers such as chitosan, cationic starch, and cellulose has been reported, emphasizing these polymers' universal availability, nontoxicity, biodegradability and low price (Yang *et al.,* 2016).

The cationic polyelectrolyte biopolymer, chitosan, has been efficiently utilized in the coagulation of organic and biological contaminants in wastewater treatment in addition to the flocculation of algae, bacteria and other applications (Divakaran and Pillai, 2002; Chen *et al.,* 2014; Wan *et al.,* 2015; Darwesh *et al.,* 2018). Several studies have reviewed the utilization of chitosan for the

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harvesting of microalgae (Chen *et al.,* 2014; Matter *et al.,* 2016; Yang *et al.,* 2016; Shuba and Kifle, 2018).

The microalgal cell morphology, its cell wall composition or structure, and its extracellular polymeric substances (EPS) are varied during different growth phases. These changes may affect the flocculation efficiency of the growing cells (Danquah *et al.,* 2009). Thus, the growth phase is one of the parameters that affect the harvesting of microalgae. Choi *et al.,* (2006) revealed that the precipitation rate of algae is significantly elevated in the stationary growth phase. Furthermore, the settling of *Scenedesmus* sp. was very little over a two-hour period in the exponential growth phase (day 6), while the highest sedimentation efficiency was recorded after fifteen days in the stationary phase (Manheim and Nelson, 2013). Emphasizing the previous reports, Danquah *et al.,* (2009) found that microalgae was settled down during the earlier growth phase (4-10 days) which is slower than in the stationary phase (10-12 days).

In a previous study, the utilization of different chitosan concentrations for harvesting *Scenedesmus* sp. at different pH values have been examined (Matter *et al.,* 2016). Whereas harvesting of microalgae could be required at different growth stages according to their application, the main objective of the current study focused on the determination of the appropriate chitosan concentration(s) for efficient flocculation of *S. obliquus* at different growth phases. The influence of different pH values and cell densities on microalgal flocculation using chitosan at different growth phases were also investigated in this study as well.

2. Materials and Methods

2.1. Microalgal Strain and Cultivation Conditions

Scenedesmus obliquus NRCIbr1 (KY621475) was previously isolated and identified (Eida *et al*., 2018). The microalga was maintained on Bold Basal Medium (BBM) (Barsanti and Gualtieri, 2014), while it was cultivated on modified Bold Basal Medium where urea was used as a sole nitrogen source instead of sodium nitrate (Amin *et al.,* 2013). An air bubble photobioreactor with continuous illumination (white fluorescent light at the intensity of 2000 Lux) was used for the cultivation of microalgae. The cultures were collected at different growth phases for the harvesting processes. The three stages of harvesting are: the late log phase (after ten days), the early stationary phase (after fifteen days) and late stationary phase (after twenty days). These growth stages for *S. Obliquus* were designated according to the previous reports of Oukarroum (2016).

2.2. Preparation of the Chitosan Solution

High molecular weight chitosan was purchased from Sigma–Aldrich (Steinheim, Germany) and was used to prepare 1 % (w/v) stock solution in 1 % acetic acid.

2.3. Harvesting Experiment

All harvesting experiments were performed in 20 mL test tubes containing 10 mL of microalgal cultures. Before dispensing the culture in tubes, the optical densities (OD_{680nm}) of the cultures, at different growth phases, were adjusted to be around 1, 2 and 3 by centrifugation or dilution with water. The pH was also adjusted to 6, 7, 8, 9 and 10 using 5 N sulfuric acid or 2 N sodium hydroxide solutions. Specific amounts of chitosan solution were added to deliver the targeted concentrations (10, 20, 40, 60 and 80 ppm) to the culture tubes. Then, the mixtures were subjected to five seconds of vortexing. After mixing, the microalgal cells were allowed to settle down for one hour before sampling.

2.4. Flocculation Efficiency

The Flocculation efficiency was measured by taking 2 ml of the samples from the middle of each sample tube at the same level to measure the optical densities at 680 nm (OD_{680nm}) using the spectrophotometer (SHIMADZU UV-2401PC, Japan). Flocculation efficiency (FE) was calculated by comparing the initial turbidity (OD_{680nm}) of the cultures with that measured after sedimentation (Farid *et al.,* 2013).

2.5. Statistical Analysis

The results were expressed as mean of three measurements ± standard division. The one-way ANOVA test and post hoc test Duncan's test (for multiple comparisons) were performed using SPSS v.20 software (IBM-SPSS, Chicago, IL, USA).

3. Results

The flocculation efficiency (FE) was studied to indicate the harvesting of *S. obliquus* induced by chitosan as a natural flocculating agent at different growth stages, culture densities and pH values.

3.1. Flocculation Efficiency of S. obliquus at Late Log Growth Phase

Figure 1 demonstrates the flocculation efficiency using different chitosan levels for *S. obliquus* cultures at late log growth phase under different pH values and culture OD_{680nm} adjusted to 1, 2, and 3. At culture OD_{680nm} adjusted to 1, the application of different chitosan concentrations (0-80 ppm) showed low flocculation efficiency (FE) percentage (lower than 38.00 %) at pH values below 10 (Figure 1a). At pH 10, a significant difference in FE was detected among all chitosan treatments, and the maximum FE value (76.60 %) was achieved when 80 ppm chitosan was applied. Through this growth stage, FE % generally trended to decline with increasing the chitosan concentrations. These results were confirmed at different studied pH values except pH 10 which displayed a significant increase in FE % when rising the chitosan concentration.

The harvesting efficacy at the late log growth phase for cultures with cells density of $OD_{680nm} \approx 2$ was evaluated at different pH and chitosan concentrations (Figure 1b). The obtained data exhibited general increase in FE % with doubling the culture density. Similar to the tendency noticed in $OD_{680nm} \approx 1$, the 80 ppm chitosan treatment at pH 10 revealed an FE % reaching 89.07 %. On the other hand, the highest harvesting efficiency at the neutral and slightly acidic conditions (pH 6 and 7) was obtained through applying 10 and 20 ppm chitosan followed by a decline in FE % when increasing the chitosan rates. However, the maximum flocculation efficiency at pH 8 reached 70.63 and 67.63 % at 20 and 40 ppm chitosan, respectively without significant difference. Nevertheless,

the 69.67 % efficiency was reached when 40 ppm chitosan was applied at pH 9.

Moreover, increasing the culture density of *S. obliquus* to OD_{680nm} ≈3 was accompanied by a further increase in the flocculation rate at different chitosan concentrations within all manipulated pH values except pH 10 which showed lower FE % (Figure 1c). Increasing the pH of the culture required a higher chitosan concentration. Twenty ppm of chitosan accomplished 83.20 % and 88.50 % flocculation rate at pH 6 and 7, respectively, while pH 8 and 9 required 40 ppm of chitosan to achieve 78.20 % and 83.2 % flocculation efficacy, respectively. Furthermore, up to 80 ppm chitosan were needed to harvest 79.00 % of *S. obliquus* cells from the culture with OD_{680nm} ≈3 and pH 10 during the late log phase. The highest flocculation rate (88.48 %) at the late log phase and OD_{680nm} ≈3 was recorded for a 20 ppm of chitosan and pH 7.

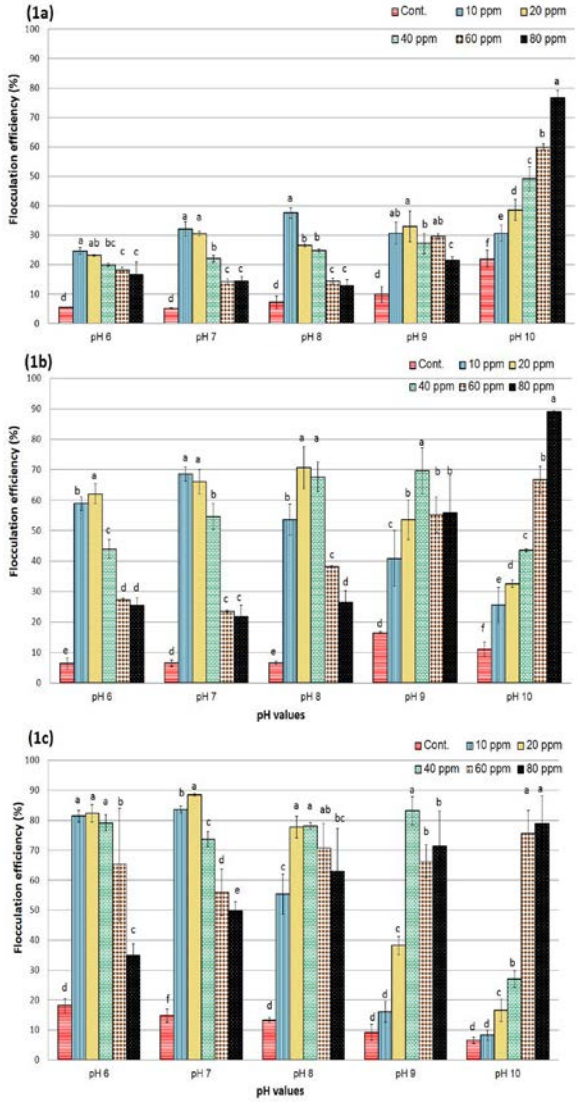

Figure1. *Scenedesmus obliquus* flocculation efficiency by various chitosan concentrations at different pH values and culture cells densities (OD_{680nm} ≈1, "1a"; OD_{680nm} ≈2, "1b" and OD_{680nm} ≈3, "1c") at late log phase. Bars are average ± standard deviation of three experiments. One-way ANOVA and post hoc test Duncan's test were done. Different letters represent significant differences (P< 0.05).

3.2. Flocculation Efficiency of S. obliquus at Early Stationary Growth Phase

The flocculation efficiency of *S. obliquus* at the early stationary growth phase was studied using different concentrations of chitosan at different growth condition including culture pH and density. The flocculation efficiency of chitosan concentrations at this growth stage differed according to cell densities and pH values as presented in Figure 2. The obtained data revealed that chitosan concentrations significantly increased the FE % at different pH values and culture densities.

At the lowest studied cells density (OD_{680nm} ≈ 1), the flocculation efficiency of algal cells using chitosan improved with the increase in cultural pH value up to 9 (Figure 2a). The highest flocculation efficiency (69.93 %) was recorded for 40 ppm of chitosan treatment at pH 9 then declined with raising the chitosan level. When the cultural pH was neutral or marginally acidic, 20 ppm of chitosan was statistically a superlative treatment although the FE % did not exceed 30 %. Additionally, once the cultural pH was adjusted to 10, the FE % increased significantly with increasing the chitosan concentration to reach its maximum value (65.21 %) after the addition of 80 ppm of chitosan. During this growth stage, the auto-flocculation (0 chitosan) showed the lowest sedimentation values (didn't exceed 18.84 %).

Increasing the cell density of *S. obliquus* culture up to OD_{680nm} ≈2 resulted in a slide increase in flocculation efficacy although the FE was trended similar to OD_{680nm} ≈1 (Figure 2b). When the pH value of the culture was lower than 9, the concentration of 20 ppm of chitosan displayed significantly greater FE % followed by a gradual decline when increasing the chitosan concentration. At this chitosan concentration, the recorded FE reached 45.47, 55.25 % and 51.50 % at pH 6, 7, and 8, respectively. Like at OD_{680nm} ≈1, 40 ppm chitosan at pH 9 exhibited the highest flocculation efficacy (70.00 %). At high alkaline condition (pH 10), the FE % was inferior to its corresponding chitosan rates at lower pH values and did not surpass 42.42 %. There was no significant difference in FE % at 0, 10, and 20 ppm chitosan in addition to the insignificancy found among 40, 60, and 80 ppm chitosan at pH 10.

The effect of chitosan concentrations on the flocculation efficiency of *S. obliquus* with cells density adjusted to OD_{680nm} ≈3 (during the early stationary growth phase) at different pH values is presented in Figure 2c. The illustrated data revealed that the FE % in cultures with OD680nm ≈3 at designated pH was higher than its value at corresponding pH level in cultures with OD680nm ≈1 and OD680nm ≈2 except at pH 10. At this culture density, the application of 20 ppm chitosan showed the maximum flocculation efficiency at pH 6 (68.90 %), although the 40 ppm chitosan at pH 7, 8 and 9 retained superior to other treatments achieving 64.90 %, 72.80 % and 77.65 % harvesting effectiveness, respectively. Increasing chitosan concentration above 40 ppm significantly decreased the FE % except at pH 10 where the opposite trend occurred. Overall, the culture with pH 10 showed the bottommost flocculation efficiency (not more than 39.22 % at 80 ppm chitosan). It was notable that increasing the culture density from OD_{680nm} ≈1 to OD_{680nm} ≈3 at pH 10 reduced the effectiveness of chitosan in the harvesting process.

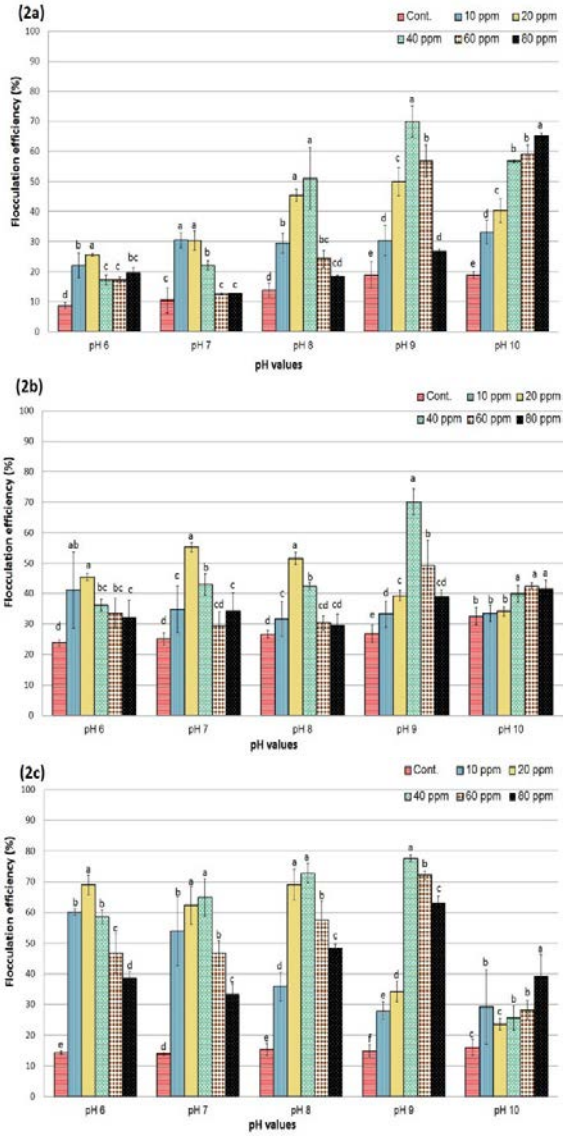

Figure 2. *Scenedesmus obliquus* flocculation efficiency by various chitosan concentrations at different pH values and culture cells densities (OD_{680nm} ≈1, "2a"; OD_{680nm} ≈2, "2b" and OD_{680nm} ≈3, "2c") at late early stationary phase. Bars are average ± standard deviation of three experiments. One-way ANOVA and post hoc test Duncan's test were done. Different letters represent significant differences ($P< 0.05$).

3.3. Flocculation Efficiency of S. obliquus at Late Stationary Growth Phase

The data acquired from the utilization of chitosan for harvesting the microalgal isolate *S. obliquus* during the late stationary phase revealed a further increase in the flocculation efficacy at the neutral, moderate acidic and moderate alkaline pH values (figure 3).

The ability of chitosan to coagulate *S. obliquus* declined generally when cultures tended to alkalinity. It was observed that during this growth stage (at different cultural ODs), increasing the pH value led to the increasing of the auto-flocculation although it retained the lowest flocculation efficacy using different chitosan concentrations.

Concerning the FE at culture OD_{680nm}≈1, the application of 10 ppm chitosan to the microalgal culture at pH 6 was the leading treatment (FE= 62.61 %) amongst all chitosan rates and pH values (Figure 3a), whereas, increasing the chitosan concentration was combined by a drop in the flocculation rate at the same conditions. Converting the culture reaction toward alkalinity required higher concentration of chitosan to achieve a reasonable FE. Regarding the culture with pH 7, the highest FE (51.78 %) was achieved using 20 ppm of chitosan while it was slightly higher (52.00 %) when 60 ppm chitosan was applied to the culture with pH 8. At pH 9, the harvesting rate of 40 ppm chitosan reached 61.68 % and was significantly superior to other chitosan concentrations. Similar to the results of FE at the early stationary phase especially at OD_{680nm} ≈2 and OD_{680nm} ≈3, the lowest harvesting efficiency with chitosan in the late stationary growth phase was found at pH 10, which mostly peaked (35.90 %) at 80 ppm of chitosan.

Furthermore, increasing the culture's OD to reach the value of around 2, the addition of 20 ppm of chitosan displayed a harvesting efficiency significantly exceeding other concentrations at pH 6 and 7 which was followed by a decline in FE when increasing the chitosan rate. The highest flocculation efficiencies (81.68 % and 81.19 %) were achieved in cultures adjusted at pH 6 treated with 20 ppm of chitosan and the culture with pH 9 treated by 60 ppm of chitosan. Correspondingly, the least flocculation efficiency (33.94 %) was observed at pH 10 utilizing 60 ppm of chitosan (Figure 3b).

However, as a result of condensing microalgal cells to OD_{680nm} ≈3, the optimum harvesting efficacy was recorded at pH 6 (81.08 %) followed by pH 7 (77.88 %) when 20 ppm and 40 ppm of chitosan were added, respectively. In the above conditions, the FE % decreased significantly with stepping up the chitosan levels at pH 6 and insignificantly at pH 7. On the other hand, the increment of cultural pH demanded higher chitosan concentrations to perform a reasonable FE (70.61 % and 74.90 % at 80 ppm of chitosan and pH 8 and 9, respectively). Similar to the above-mentioned culture density, the lowest flocculation efficiency was observed at pH 10; even the highest chitosan concentration (80 ppm) did not harvest more than 22.83 % of *S. obliquus* cells from the culture medium (Figure 3 c).

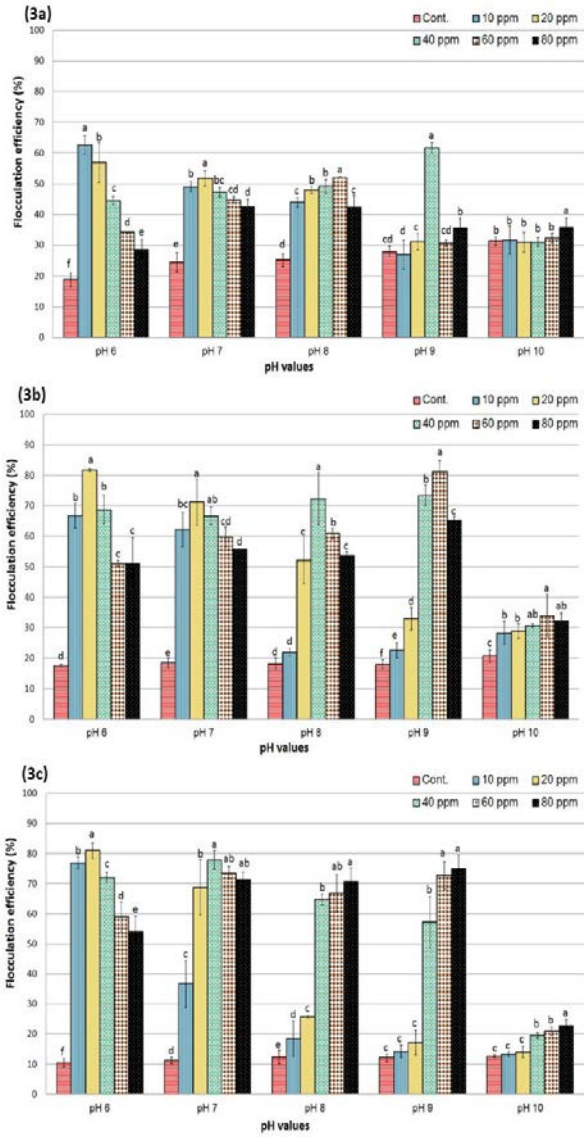

Figure 3. *Scenedesmus obliquus* flocculation efficiency by various chitosan concentrations at different pH values and culture cells densities (OD_{680nm} ≈1, "3a"; OD_{680nm} ≈2, "3b" and OD_{680nm} ≈3, "3c") at late stationary phase. Bars are average ± standard deviation of three experiments. One-way ANOVA and post hoc test Duncan's test were done. Different letters represent significant differences (P< 0.05).

4. Discussion

The evaluation process of utilizing various chitosan concentrations to flocculate microalgae at the late log growth phase, and early and late stationary phases is performed in this study. The performance of chitosan concentrations was determined in *S. obliquus* cultures adjusted to different pH values (6-10) and cell densities (OD_{680nm} ≈1-3) at each growth stage. In a previous study of the bio-flocculation of *S. obliquus* grown at the early logarithmic growth phase, chitosan was proven to be an effective bio-flocculating agent and the pH was found to be a major influencing factor to determine its efficacy (Matter *et al.*, 2016). Microalgal harvesting stages generally differ according to the purpose of biomass uses (Chang and Lee, 2012; Khanra *et al.*, 2018).

The obtained results revealed low sedimentation efficacy (5.5 % - 32.49 %) when chitosan was not applied (control) at all pH values and at all growth stages. In this case, the highest precipitation percentages of the studied microalgae were recorded at the highest pH values. In spite of the limited available information concerning the autoflocculation, Ummalyma *et al.* (2016) suggested the self-production of flocculating agents (e.g., poly-saccharides and glycoprotein) or a bridge-forming ability between algal cells through charge neutralization with modifying culture pH. Similar results were stated by Gerchman and the co-authors (Gerchman *et al.*, 2017).

Chitosan is a promising flocculating agent for coagulating microalgae harvesting, but its optimized application conditions are not steeled so far (Sajjad *et al.*, 2017; Matter *et al*., 2018). Generally, the application of various rates of chitosan improved the flocculation efficiency at different growth phases, culture densities, and pH values. The most consistent mechanism for harvesting microalgae using chitosan is the electrostatic interaction between its amino groups with the negatively charged groups (e.g. amide and carboxylic groups) on the algal cell surface (Pranowo *et al*., 2013) leading to adsorption and charge neutralization (Tran *et al.*, 2013). This fact was supported by the results of Lu *et al.* (2017) who studied the changes in Zeta potential during chitosan flocculation and their results indicated that the adsorption-bridging and charge neutralization were the essential mechanisms for algal flocculation.

The obtained data obviously showed that the FE increased with increasing the chitosan concentration at all growth phases and different pH levels to reach a maximum then a decline with any further increase in the chitosan dose. These results may refer to the charge neutralization and bridging phenomena and the decline in harvesting ability with chitosan over application could rely on the role of excess amino groups in the restabilization of the cells resulting in a decrease in the coagulation percentage (Yang *et al.*, 2016; Yunos *et al.*, 2017). Similar results were stated by Rashid *et al.* (2013) who reported that the application of chitosan overdose dramatically reduced the harvesting efficiency. It was reported that the coagulation efficacy of microalgae utilizing chitosan as a flocculating agent is very sensitive to pH (Rashid *et al.*, 2013; Xu *et al.*, 2013; Matter *et al*., 2018)

Results of the current study exhibited that while 10 to 20 ppm of chitosan achieved the greatest flocculation efficiency at neutral and mild acidic pH (6-7), cultures with higher pH (8-9) required superior chitosan concentrations (20 to 40; even 60 ppm) to flocculate reasonable amounts of *S. obliquus.* The advanced capability of chitosan in the bioflocculation process at a low pH could be explained by its enhanced charge neutralization and bridging effects at this condition (Yang *et al.*, 2016). In this respect, Xu *et al.* (2013) indicated that reducing the cultural pH can decrease the required chitosan concentrations to motivate an effective flocculation.

In accordance with our data Wu *et al.* (2012) reported that at the same pH value, the harvesting efficiency increased with increasing the biomass concentration which increased with the culture aging. In another study which agreed to these results, it was observed that at high pH

value (10), a general decrease in the flocculation efficiency was noticed with increasing the culture density. The increase in the harvesting percentage could be explicated by the fact that, high opaque algal cultures have higher negative charge that can strongly attach to positively charged biopolymers such as chitosan and neutralize its positive surface charge (Farid *et al*., 2013).

5. Conclusion

The current study concludes that different chitosan concentrations could be required for achieving a better and economical harvesting of *S. Obliquus* by bioflocculation. Harvesting *S. Obliquus* using chitosan for producing bioactive products as well as biofuels is depending on growth phases, cultural pH, and cell densities. The efficacy of chitosan in harvesting *S. obliquus* could be improved by selecting the most suitable concentration according to the harvesting conditions (pH, cells density and growth phase). The recommended chitosan concentrations (ppm) for the best harvesting of *S. obliquus* under the study conditions are summarized in table (1).

Table 1. Recommended chitosan concentrations for the best bioflocculation of *S. obliquus* at different growth conditions.

	Growth Phase								
	Late log phase			Early stationary phase			Late stationary phase		
pH	OD 1	OD 2	OD 3	OD 1	OD 2	OD 3	OD 1	OD 2	OD 3
6	10	20	10	20	10	20	10	20	20
7	10	10	20	10	20	20	20	20	40
8	10	20	20	20	20	20	60	40	60
9	10	40	40	40	40	20	40	60	60
10	80	80	60	80	40	80	80	60	80

In the table, when there is no significant difference in flocculation efficiency between two chitosan concentrations, the recommended chitosan level was the lower concentration.

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The Impact of the Complexity of Cystic Fibrosis in Jordanian Patients on the Spectrum of Cystic Fibrosis Transmembrane Conductance Regulator Mutations

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Abstract

Contrary to earlier beliefs, cystic fibrosis (CF) is relatively common in Arab populations with an estimated incidence of about 1/2500 live births in Jordan. In order to identify the common mutations among CF Jordanian patients a total of 386 Jordanian CF patients (323 families) were followed up over a period of fifteen years from diagnosis and were screened for Cystic Fibrosis Transmembrane Conductance Regulator (*CFTR*) mutations. Furthermore, to characterize the spectrum of the *CFTR* mutations, DNA samples were obtained from sixty-eight patients and sixty-six parents and were subjected to complete *CFTR* gene screening by multiplex heteroduplex (mHET) analysis followed by direct sequencing. The screening included promoter, all exons with flanking intron sequence (including T-tract in intron 8) and resulted in the identification of twenty-six different mutations. The most prevalent mutation, p.Phe508del was found to account only for 7.4 % of the identified *CFTR* mutations. This low frequency of the p.Phe508del mutation among Jordanian patients is comparable with native Asians. In this study, seven *CFTR* mutations, which have not been previously reported, were identified (c.CFTR dele2 (ins186), c.296+9A>T, c.297-10T>G, p.Thr388Met, p.Thr760Met, c.3670delA and c.4006delA). The large number of mutations reflects the ethnic diversity of the Jordanian population and the complex history of the country. The obtained results will assist to improve the understanding of the molecular basis of the pathophysiology of cystic fibrosis, genetic counseling, and prenatal diagnosis in Jordan. Additionally, it will identify the correlation between the *CFTR* genotypes and the CF phenotypes in the Jordanian population, especially among the newly discovered mutations, which will, in turn, broaden the management of the disease in Jordan.

Keywords: Cystic fibrosis, Mutations, Genotypes, Jordan

1. Introduction

Cystic fibrosis (CF) is the most common lethal autosomal recessive disorder in Caucasians with the incidence of one in 2000 to 3000 live births in various populations (Tzetis *et al.,* 1996). The incidence rate of CF is considerably higher in certain ethnic groups such as Celts in Brittany (western France) where it is among the highest in the world with an estimated frequency of 1: 1983 (Scotet *et al.,* 2012) in contrast to Japan which has the low incidence rate of estimated frequencies of 1: 350,000 live births (Bois *et al.,* 1978; Rosenfeld *et al.,* 1997; Yamashiro *et al.,* 1997; Padoa *et al.,* 1999). Data from the annual report of US 'Cystic Fibrosis Foundation Registry' revealed an estimated 29497 CF patients in the United States and more than 70000 patients worldwide. In the Arab world, CF disease was initially thought to be a very rare disease; however, studies revealed that CF incidence rates are estimated at 1: 2500 live births in Jordan (Nazer, 1992; Kakish, 2001) followed by 1:5000 in Bahrain (Al-Mahroos, 1998) which is comparable with the incidence of CF among Caucasians ranging between 1:2000 and 1:4000 live births. (Dawson and Frossard, 2000; Nazer, 1992).

Cystic Fibrosis is caused by mutations in the cystic fibrosis transmembrane regulator (*CFTR*) gene encoding the c-AMP-activated chloride channel (Linsdell, 2006). The actual spectrum of *CFTR* mutations varies among different ethnic groups and geographical locations. More than 1900 mutations and variants in the *CFTR* gene have been reported from various populations (Jonsdottir *et al.,* 2008). The most frequently detected mutation in these populations is p.Phe508del. According to these reports, p.Phe508del mutation does not seem to account for more than 20 % of all Caucasian CF chromosomes (Antiñolo *et al.,* 1997; Lucotte *et al.,* 1995). In the Arab countries, several types of research have been investigating the

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mutational patterns of *CFTR* genes amongst patients from Oman (Frossard *et al.,* 1998), Bahrain (Al-Mahroos, 1998), Saudi Arabia (Nazer *et al.,* 1989; Kambouris *et al.,* 2000), United Arab Emirates (Saleheen *et al.,* 2006), Jordan (Al-Batsh *et al.,* 2013), Qatar (Rahman *et al.,* 2006), and Libya (Hadj Fredj *et al.,* 2011).

A large group of Arab CF patients including 202 patients with a follow up of more than nine years have been reported (Rawashdeh and Manal, 2000). Around 73 % of the patients were reported with classical CF clinical manifestations (growth failure, malabsorption, and pulmonary involvement). Some of the less common CF-associated clinical features, such as liver disease and pseudo-Bartter syndrome were significantly more prevalent among Jordanian patients than Caucasian populations (13.6 % vs. 3-5 % and 7.4% vs. few cases report respectively). These clinical differences could be attributed to both genetic and environmental factors. Some mutations are associated with certain clinical features and sweat chloride concentrations (Beck *et al.,* 1999; Bienvenu *et al.,* 1996; di Sant'Agnese *et al.,* 1953; Romey *et al.,* 1999; Southernl and Peckham, 2004; Wilschanski *et al.,* 1995; Braun *et al.,* 2006; Chaudry *et al.,* 2006; Hamosh *et al.,* 1998).

Genetic investigations of CF have not been conducted on Jordanian population, which is considered as a population of interest for many reasons. The territory constituting modern Jordan was the site of some of the earliest settlements known to the world (Ammonites and the kingdoms of Edom, Gilead, and Moab). The population of Jordan consists almost entirely of Arabs along with some racial minorities of Circassians and Armenians (Al-Kindy *et al.,* 2014). Furthermore, the consanguinity rate in Jordan varies from 50 % to 64 %; therefore, it is an important feature for the occurrence of genetic diseases among this population (Al-Salem and Rawashdeh, 1993).

Accurate information on the incidence and the spectrum of mutations are considered essential for health care planning and predicting the risk for prenatal diagnosis in Arab countries. Therefore, this study is aimed at identifying the spectrum of the *CFTR* mutations and polymorphisms among the Jordanian population.

2. Materials and Methods

2.1. Patients

A total of 386 patients from 232 families were diagnosed with CF. The diagnosis of CF was based on either a suggestive or consistent clinical picture with CF as well as concentrations of two sweat sodium and chloride tests with 60mmol^{-1} or higher (Doull *et al.,* 2001; LeGrys, 1996). The presence of other affected family members, the death of a sibling with a similar condition, the degree of parent's consanguinity, family pedigrees, sweat chloride concentration and relevant clinical information were collected at diagnosis and during follow ups.

2.2. Blood Sampling and DNA Extraction

Informed consents were signed by all participants in the study. EDTA blood samples were collected from 150 patients and their parents for mutation screening. Plasma was separated by centrifugation, and stored at -80^{o}C. Genomic DNA was extracted from blood by a standard method and was subjected to *CFTR* mutation screening analysis.

2.3. Screening for CFTR Mutations

Genomic DNA from patients was subjected to mutation detection analysis using *CFTR* -specific primers previously described (Zielenski *et al.,* 2002) applying polymerase chain reaction (PCR) and multiplex heteroduplex (mHET) analysis on the Hydrolink gel matrix and direct sequencing using the Thermo Sequenase radiolabeled terminator cycle sequencing kit (USB) which has been refined by (Zielenski *et al.,* 2002). In addition, the thymidine tract (T-tract) in the acceptor splice site in intron 8 was evaluated to detect the RNA splice variant c.IVS8-5T (Mak *et al.,* 1997).

3. Results

The diagnosis of CF was conducted on 386 (220 boys and 166 girls) patients coming from 232 families. Consanguineous marriage was present in 263 patients (68 %); the parents were first or second-degree cousins in 205 patients (53 %). Complete clinical data were available on 294 patients (Table 1). DNA samples from sixty-eight patients and sixty-six parents were obtained for the study and screening was completed for sixty-eight CF patients. The selected sixty-eight patients were born from healthy non-consanguineous parents.

Twenty-six different *CFTR* mutations/variants accounting for 45.7 % of the CF chromosomes were identified. The spectrum of *CFTR* mutations identified is shown in Table 2. The p.Phe508del mutation accounted for only 7.4 % in this cohort. Only four patients were homozygotes for p.Phe508del mutation with sweat chloride concentration being above 100 mmol/l, pancreatic insufficiency (PI) and severe lung disease. Two patients died; one patient died at the age of three years from respiratory failure, and the second was aged thirteen years and died from liver failure.

Four patients were p.Phe508del heterozygotes. They had a very variable clinical presentation and disease severity probably due to the effect of the second mutation, despite the fact that all were diagnosed with pancreatic insufficiency. Among the mutations detected, seven alleles were found for the first time [c. CFTR del2 (ins186), c.296+9A>T, c.297-10T>G, p.Thr388Met, p.Thr760Met, c.3670delA and c.4006delA]. These mutations, as well as short clinical characteristics of the patients carrying them, are shown in Table 3.

Seven different *CFTR* mutations were identified in nine chromosomes of twelve patients with overt liver disease (p.Phe508del, c.CFTR del2, c.296+9A>T and c.297-10T>G, c.1716G>A, including c.1677delT and c.IVS8-5T). The liver disease was not associated with any specific genotype. *CFTR* mutations were identified in six alleles of four patients in the cohort who had hypotonic dehydration associated with hyponatremia, hypochloremia, hypokalemia, and metabolic alkalosis (Pseudo-Bartter syndrome). One patient was homozygote for p.Gln1100Pro, and a second was homozygote for *CFTR* del2. Other mutations detected in these patients were p.Phe508del and p.Gly1244Glu. None of them carried the p.Thr338Ile and p.Ser1455X mutations, previously associated with these manifestations (Padoan *et al.,* 1996; Epaud *et al.,* 2005).

Table1. Major presenting symptoms in 294 Jordanian children with cystic fibrosis

CFTR mutation/variant	Locationexon/intron	Number of chromosomes	Proportion (%)
p.Phe508del	10	10	7.4
c.1677delTA	intron 10	6	4.4
c.IVS8-5T	intron 8	5	3.7
c.CFTR del17a-18	17a-18	4	3.0
*c.3670delA	19	4	3.0
c.CFTR dele2	2	4	3.0
p.Arg75Gln	3	2	1.5
c.1716G>A	10	2	1.5
p.Arg1066Cys	19	2	1.5
p. Gln1100Pro	19	2	1.5
p.Trp1282X	20	2	1.5
c.3849+5G>A	intron 19	2	1.5
*c.CFTR del2(ins186)	2	2	1.5
*c.296+9A>T	intron 2	2	1.5
*c.297-10T>G	Intron 3	2	1.5
p.Ala120Thr	4	1	0.7
p.Ile148Thr	4	1	0.7
*p.Thr388Met	8	1	0.7
*p.Thr760Met	13	1	0.7
p.Asp1152His	18	1	0.7
p.Ser1235Arg	19	1	0.7
p.Gly1244Glu	20	1	0.7
c.3849+10kbC>T	Intron 19	1	0.7
p.Asn1303Lys	21	1	0.7
*c.4006delA	21	1	0.7
p.Gly1244Asp	20	1	0.7
Total identified		62	45.7
Unknown		74	54.3
Number of tested chromosomes		136	100

*NOT previously reported mutation

Table2. The spectrum of *CFTR* Mutations among Jordanian CF population

Clinical manifestations	Number of patients	Percentage (%)
Respiratory	54	18.4
Malabsorption	53	18
Gastrointestinal & Respiratory	108	36.6
Meconium ileus	18	6
Liver disease	40	13.6
Pseudo-Bartter syndrome	21	7.4
Total	294	100

Table 3. Genotypic and phenotypic matching of new *CFTR* mutations in Jordanian population.

Mutation	Nucleotide Change	Exon/ Intron	Consequence	Second mutation	Age at DX (mo)	Lung Disease	Pancreatic Insufficiency/ Pancreatic sufficiency/ (PI/PS)	Sweat Cl Mmol/L	Other Symptoms
c.CFTR del2(ins186)	Complex	2	Large del.	Unknown	15	Moderate	PI	65	
c.296+9A>T	IVS 2	Missplicing	splicing	c.296-10T>G	72	Severe	PI	65	Hepatomegaly
c.297-10T>G	IVS 2	Missplicing	splicing	c.296+9A>T	72	Severe	PI	65	Hepatomegaly
p.Thr388Met	T to ?/ a 1295	8	Missense	p.Trp1282X	11	Moderate	PI	80, 90	Normal growth
p.Thr760Met	C to T at 2411	13	Missense	p.Phe508del /c.1677delTA	60	Moderate	PI	110	
c.3670delA		19	Frameshift	c.3670delA	4	Severe	PI	112	Severe
c.4006delA		21	Frameshift	p.Phe508del	14	Mild	PI	95	Malnutrition

4. Discussion

A large number of CF patients points to the high prevalence of this disease in Jordan. The incidence rate of CF in Jordan is 1: 2500 live births; this is close to the reported rate of Caucasian patients (Singh *et al.,* 2015; Nazer, 1992). The high consanguinity rate, the large (average of 6.7 individuals) and the extended families in the Jordanian community can explain the segregation of many cases in relatively few pedigrees. The higher number of boys over girls in families in Jordan (1.3:1) may reflect the higher mortality rate among the affected females according to the observed gender gap in the CF survival rate (Rosenfeld *et al.,* 1997; Schneiderman-Walker *et al.,* 2005).

Complete *CFTR* mutation screening in the cohort of sixty-eight CF patients led to the identification of twenty-six different mutations/variants accounting for 45.5 % of tested CF chromosomes. Relatively the large number of different mutations found in less than half of the analyzed chromosomes reflects a complex history with a considerable ethnic diversity of the Jordanian population that goes back several centuries. Being at the crossroads of the Middle East, the location of the lands of Jordan have served as a strategic point connecting Asia, Africa, and Europe.

More than 1900 mutations have already been reported in the *CFTR* gene (US Cystic Fibrosis Foundation, 2010; Jonsdottir *et al.,* 2008). The frequencies of each mutation in a population vary according to the geographical and ethnic origin of the population. Reports on these mutations in Arab populations have so far been very limited. Recently, a panel of eleven common mutations accounting overall for 70 % of all Arab CF chromosomes have been reported: p.Phe508del, c.3120+1G> A, p.Asn1303Lys, p.Trp1282X, p.Gly115X, c.711+1G>A, p.Ser549Arg, p.Ile1234Val, c.1548delG, p.His139Leu and c.4010del4 (Bois *et al.,* 1978; Kakish, 2001). The latter three mutations are believed to have originated in the Arab native populations since they were never described in Caucasian CF patients (Kerem *et al.,* 1995; Kambouris *et al.,* 2000; El-Harith *et al.,* 1997; Angelicheva *et al.,* 1994).

The spectrum of mutations detected so far in Jordanian CF population is different from that reported in many countries across Europe and North America (Guilloud-Bataille *et al.,* 2000; Rock *et al.,* 2005; Tzetis *et al.,* 1996). The frequency of most common Caucasian mutation (p.Phe508del) was present only in 7.4 % of Jordanian patients. This is the lowest p.Phe508del mutation ever reported in any ethnic group (Elahi *et al.,* 2006; Kakish, 2001; Lao *et al.,* 2003). The unusual low frequency of the p.Phe508del mutation in the current study matches the same low incidence of a former study in Jordan (Al-Batsh *et al.,* 2013), in Asia (12-31 %) compared to the 66 % high frequency worldwide (Singh et al., 2015). The rarity of the p.Phe508del mutation can be attributed partly to the genetic heterogeneity among Jordanian population due to the genetic drift and gene flow that play a major role in reshaping the genetic structure of Jordanians. Patients with one p.Phe508del mutation had a wide range of clinical presentation and a variable course of the disease, although all were PI. In adult CF patients, p.Phe508del was associated with PI in only19 % of cases (Modolell *et al.,* 2001). This variability is partially related to the presence of another mutation (Antiñolo *et al.,* 1997). The second most common (4.4 %) Jordanian *CFTR* allele, is c.1677delTA deletion, which is of high prevalence in the Turkish population (Onay *et al.,* 1998). This may be associated with the four centuries of Ottoman rule (1516-1918 CE). Another ethnic-specific mutation relatively frequent (3.0%) among the Jordanian *CFTR* mutations is multi-exonic deletion spanning exons 17 to 18 (c.CFTR del17a-18) previously reported in Palestinian CF patients (Lerer *et al.,* 1999). This can be explained by the presence of the large Palestinian community in the country of Jordan. There are also other mutations including [p.Asp1152His, p.Gly1244Glu, c.3849+5GA, c.CFTR del2 (186kb) and c.CFTR del2 (5kb)] that appear to be more widely spread throughout the Near and Middle East, but are rarely observed elsewhere. Seven mutations were found for the first time in the Jordanian population [c.CFTR del2 (ins186), c.296+9A>T, c.297-10T>G, p.Thr388Met, P.Thr760Met, c.3670delA and c.4006delA]. The most frequent mutation was the c.3670delA frameshift mutation which was diagnosed at a younger age with PI, severe lung disease, and a correspondingly worse prognosis. Finally, some mutations in Jordan are

compatible with mutations in many other regions of the world, such as p.Asn1303Lys, p.Trp1282X, p.Ile148Thr, p.Ser1235Arg, c.3849+10kbCT, and p.Arg1066Cys.

The relatively common liver disease in Jordanian CF patients was not associated with a specific *CFTR* mutation (genotype). No specific genetic abnormality has been linked with more frequent or more advanced involvement of the liver and biliary tree in CF patients (Fagundes *et al.*, 2005). However, there is evidence that hepatobiliary involvement in CF correlates with pancreatic insufficiency and specific human leukocyte antigen (HLA) loci A2, B7, DR2 (DR15), and DQ6 (Fagundes *et al.*, 2005).

This large diversity in the *CFTR* mutations makes the technical feasibility of a large-scale CF carrier screening extremely difficult (Grody *et al.*, 1997). However, these results can help improve genetic counseling and prenatal diagnosis of CF as well as the understanding of the molecular pathophysiology of the disease.

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In vitro Anti-Proliferative Activity of the *Rubia tinctorum* and *Alkanna tinctoria* Root Extracts in Panel of Human Tumor Cell Lines

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Abstract

Cancer is a devastating disease and is considered number one killer worldwide. Herbal formulations had played a key role over the past several decades in the development of anti-cancer drugs. Medicinal plants, which are endemic in Jordan, are known for several biological activities in particular their anti-cancer activity. However, the anti-cancer efficacy of the root extracts of Jordanian *Rubia tinctorum* and *Alkanna tinctoria* is not yet reported. To address this issue, this study assessed the anti-cancer activity of some root extracts obtained from Jordanian *R. tinctorum* and *A. tinctoria* in different tumor cell lines including the tongue, bladder, colon, gastric, lungs, breast, pancreas, and renal tissue origins by modified propidium iodide (PI) based monolayer assay. Among the tested root extracts obtained by different solvent systems, *A. tinctoria* in 100 % ethanol and methanol showed prominent anti-cancer activity against MDA-MB-231breast cancer cells (IC_{50}: 2.98 µg/ml, IC_{70}: 6.03 µg/ml), and CAL-27 tongue squamous carcinoma cells (IC_{50}: 3.86 µg/ml, IC_{70}: 5.97 µg/ml) respectively. Different solvent root extracts of *R. tinctorum* exhibited a similar trend of anti-tumor activity in both CAL-27 and MDA-MB-231 cells. The anti-proliferative property of the extracts on CAL-27 and MDA-MB-231 cells is unclear. However, it can be concluded that the observed anti-cancer potential can be attributed to the phenolic compounds of the extracts as high polar solvents were used for extraction. The current study forms the rationale for isolating significant amount of anti-cancer active compounds from *R. tinctorum* and *A. tinctoria*.

Key words*: Rubia tinctorum, Alkanna tinctoria,* Anti-cancer, CAL-27, MDA-MB-231

1. Introduction

Cancer is a major public health burden in both developed and developing countries. According to the American Cancer Society, deaths arising from cancer constitute 2-3 % of the annual deaths recorded worldwide. In the United States, about 600,920 Americans were expected to die of cancer in 2017, which translates to about 1650 people per day, and this is expected to be on the rise in 2018 (Siegel *et al*., 2017). According to the morbidity and mortality rates associated with this disease, in addition to the critical economic burden, there are no effective strategies for the development of anti-cancer drugs. The emerging drug resistance concerning the existing cancer chemotherapy is another major hurdle to overcome in order to achieve therapeutic efficacy. Therefore, finding new drugs or drug formulations is highly warranted to alleviate the above-mentioned hurdles. Historically, plants have been the primary sources of natural products for drug discovery including plant-derived agents, such as vinblastine (VBL) and vincristine (VCR), etoposide, paclitaxel (Taxol), docetaxel, topotecan, and irinotecan, which are amongst the most effective cancer chemotherapeutic agents (Cragg *et al*., 2012).

In the Hashemite Kingdom of Jordan, natural sources consisting of more than 2500 wild plant species from 700 genera exist; of these, there are approximately 100 endemic species, 250 rare species, and 125 very rare species (Al-Eisawi *et al.*, 2000; Oran and Al Eisawi, 2014). Traditional medicine practices are part of the Jordanian culture, and there seems to be a wealth of ethnobotanical studies providing a new major contribution in the search for invaluable phyto-pharmaceuticals or the development of functional foods or nutraceuticals (Al-Khalil, 1995). Literature surveys based on published studies indicated that in Jordan and the neighboring countries, twenty-seven plant species are considered as traditional remedies for the treatment of different types of cancers (Hudaib *et al.*, 2008). Ethanolic extracts of more than seventy medicinal herbs from the Jordanian flora, belonging to sixty-seven species and thirty-four families,

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were evaluated for their anti-proliferative activity on a breast cancer cell line including *Alkanna* and *Rubia* genus (Abu-Dahab and Afifi, 2007). *Alkanna tinctoria* (Boraginaceae) and *Rubia tinctorum* (Rubiaceae) are widespread in the local regions of Jordan. Alkannin is a well-known phenolic compound present in *A. tinctoria* roots, and is reported for numerous biological activities, such as free radical scavenging (Kourounakis *et al.,* 2002), and anti-inflammatory properties (Kourounakis *et al.*, 2002). Alkannin has been reported to suppress the UV radiation induced apoptosis in human keratinocytes by modulating caspase and HSP70 signaling (Yoshihisa *et al.*, 2012). Naphthoquinones isolated from the root extract of *A. tinctoria* induces apoptosis and cell cycle arrest in colorectal cancer cells (Tung *et al.,* 2013). Assimopoulou *et al*. reported that hydroxynaphthoquinones and other metabolites have been isolated from ten species of the genus *Alkanna* (Assimopoulou *et al.,* 2006). The genus *Rubia* is known to be a source of several anthraquinones, and it is reported for different pharmacological activities such as anti-cancer, anti-microbial, anti-fungal, and anti-oxidant activities (Park *et al.,* 2009). The root extract of *R. tinctorum* is effective against kidney and bladder stones (Blomeke *et al.,* 1992; Westendorf *et al*., 1998). The trihydroxy-anthraquinones alizarin and purpurin isolated from hairy root cultures of *R. tinctorum* inhibited the proliferation, adhesion and migration of melanoma cells (Eszter Lajko *et al.,* 2015).

A. tinctoria and *R. tinctorum* are well reported for anti-cancer activity. However, the anti-cancer efficacy of the root extract of Jordanian *A. tinctoria* and *R. tinctorum* is not yet reported. As phenolic compounds are known to be effective anti-cancer active principles, high polar solvents such as methanol, ethanol and water are always recommended for the extraction of such compounds more than low polar and non- polar solvents. In the current study, we used National Cancer Institute (NCI), USA recommended panel of tumor cell lines which are widely used to study anti-cancer activity of compounds (Heidi Ledford, 2016). In this study, roots of Jordanian *A. tinctoria* and *R. tinctorum* were extracted in different solvent systems (100 % ethanol, 50 % ethanol, 100 % methanol, hot water and cold water) and the extracts were screened in a panel of tumor cell lines (tongue, bladder, colon, gastric, lungs, breast, pancreas, and renal) for anti-cancer activity.

2. Materials and Methods

2.1. Plant Material

Rubia tinctorum roots were collected from the local regions of North Badia in Jordan (3489 kilometers from Amman). *Alkanna tinctoria* roots were collected from the local regions of Ajloun (72 kilometers from Amman) in Jordan. Both plants were taxonomically identified by the botanist based on anatomy and microscopic observation of internal structure of organs. Voucher specimen was deposited in the herbarium of the Biology Department of the Faculty of Science at the University of Jordan.

2.2. Solvent Extraction

The collected plant materials were shade-dried and finely powdered by a grinding mill. Both *R. tinctorum* and *A. tinctoria* root powders were extracted with 100 % ethanol, 50 % ethanol, and 100 % methanol, and the extracts were centrifuged at a speed of 7000 rpm at 4ºC for thirty minutes. Then extracts were lyophilized at -50ºC and stored at 4˚C until use for the experiment.

2.3. Hot Aqueous Extraction

R. tinctorum and *A. tinctoria* root powders were extracted with boiling water for thirty minutes, and the extracts were kept for cooling at room temperature for four hours under stirring. Then extracts were centrifuged at 5000 rpm at room temperature, filtered and lyophilized and stored at 4˚C until use for the experiment.

2.4. Cell Lines

A Panel of tumor cell lines consists of tongue, bladder, colon, gastric, non-small cell lung, breast, pancreatic, and renal cancer. Non-PDX-derived cell lines were either kindly provided by the NCI (Bethesda, MD), or were purchased from ATCC (Rockville, MD) or DSMZ (Braunschweig, Germany), or JCRB (Japanese Collection of Research Biosources Cell Bank, Japan) (Table 1).

Table 1. Authenticated cell lines used for the study.

#	Cell line				
	Type		Name	Origin	STR Analysis
1	Bladder	BXF	RT112	DSMZ	Authentic 271
2	Colon	CXF	COLO205	NCI	Authentic
3	Gastric	GXA	MKN45	JCRB#0254	Ordered
4	Tongue	HNXF	CAL-27	DSMZ	Authentic
5	Lung	LXFA	526L	Xenograft, Freiburg	Authentic 273
6	Lung	LXFL	529L	Xenograft, Freiburg	Authentic
7	Mammary	MAXF	MDA-MB-231	ATCC	Authentic 274
8	Mammary	MAXF	SK-BR-3	ATCC, HTB-30	Authentic
9	Pancreas	PAXF	1675L	Xenograft, Freiburg	Authentic 275
10	Renal	RXF	SN12C	NCI	Authentic

JCRB: Japanese Collection of Research Bioresources ; ATCC: American Type Culture Collection, Rockville, MD, USA; NCI: National Cancer Institute, Bethesda, MD, USA; DSMZ: Deutsche Sammlung von Mikroorganismen and Zellkulturen Braunschweig, Germany

2.5. Cell Culture

Cell lines were routinely passaged once or twice weekly and maintained in culture for up to twenty passages. All cells were grown at 37°C in a humidified atmosphere with 5 % CO_2 in RPMI 1640 medium (25 mM HEPES, with L-glutamine, FG1385, Biochrom, Berlin, Germany) supplemented with 10 % (v/v) fetal calf serum (Sigma, Taufkirchen, Germany) and 0.1 mg/mL gentamicin (Life Technologies, Karlsruhe, Germany).

2.6. Anti-Cancer Assay

A modified propidium iodide (PI) based monolayer assay was used to assess the anti-cancer activity of the extracts (Dengler *et al.,* 1995). Briefly, cells were harvested from exponential phase cultures, counted and plated in ninety-six well flat-bottom microtiter plates at a cell density of 6,000 to 12,000 cells/well depending on the cell lines growth rate. After a twenty-four-hour recovery period, cells were allowed to resume exponential growth, 10 µL of culture medium (4 control wells/cell line/plate) or of culture medium with the extracts added. The extracts were applied at ten concentrations in half-log increments to 0.3 (v/v) or 100 µg mL^{-1}, and the treatment continued for four days. After four days of treatment, the cells were next washed with 200 µl PBS to remove the dead cells and debris, then 200 µL of a solution containing 7 µg/mL propidium iodide (PI) and 0.1 % (v/v) Triton X-100 was added. After an incubation period of 1-2 hours at room temperature, fluorescence (FU) was measured using the Enspire Multimode Plate Reader (excitation $\lambda = 530$ nm, emission $\lambda = 620$ nm) to quantify the amount of attached viable cells.

2.7. Calculation of IC_{50} and IC_{70}

IC_{50} and IC_{70} values were calculated by four parameters non-linear curve fit using Oncotest Warehouse Software. For calculation of mean IC_{50} values, the geometric mean was used.

2.8. Data Evaluation

An assay was considered fully evaluable if all the following quality control criteria were fulfilled. Z'-factor calculated within the assay plate ≥ 0.5 (kevorkov and Makarenkov, 2005), Fluorescence intensity of > 500 U from the untreated control wells, equivalent to a control/background ratio > 3.0. and coefficient of variation in the growth control wells ≤ 30 %.

2.9. Sigmoidal Concentration Response Curve

Drug effects were expressed in terms of the percentage of the fluorescence signal, obtained by comparison of the mean signal in the treated wells with the mean signal of the untreated controls (expressed by the test-versus-control value, T/C-value [%]):

T/C (%) = mean fluorescence signal treated group/ mean fluorescence signal control group X 100

Sigmoidal concentration-response curves were fitted to the data points obtained for each compound using four parameters non-linear curve fit (Oncotest Data Warehouse Software). IC values are reported as absolute and relative IC_{50} and absolute IC_{70} values. The absolute IC_{50} value reflects the concentration of the extracts that achieves T/C=50%. The absolute IC_{70} value gives the concentration of the extracts that achieves T/C=30%. The relative IC_{50} value is the concentration of extracts that gives a response half way between the top and bottom plateau of the sigmoidal concentration-response curve (inflection point of the curve).

3. Results

3.1. Geo Mean of Anti-Cancer Activity

The extracts were tested in half-log steps up to a test concentration of 100 µg/mL. Among the extracts tested, 100 % ethanol extract of *A. tinctoria* (geo mean IC_{50} value of 6.98 µg mL^{-1}) was most potent, followed by a 100 % methanol extract of *R. tinctorum* (Geo mean IC_{50} 10.66 µg mL^{-1}), 50 % ethanol extract of *R. tinctorum* (Geo mean IC_{50} 12.33 µg mL^{-1}) and 100 % methanol extract of *A. tinctoria* (Geo mean IC_{50} 14.91 µg mL^{-1}) (Table 2).

Table 2. The anti-cancer activity of *Alkanna tinctoria* and *Rubia tinctorum* root extracts (Geometric Mean value).

Cell line	*A. tinctoria* 100% ethanol extract (µgmL)	*A. tinctoria* 100% methanol extract (µg/mL)	*R. tinctorum* 50% ethanol extract (µg/mL)	*R. tinctorum* 100% methanol extract (µg/mL)
BXF RT112	5	6.2	4.33	2.76
CXF COLO 205	10.57	33.33	26.93	23.4
GXA MKN45	11.45	14.1	10.44	8.33
HNXF CAL-27	3.83	4.61	2.94	2.53
LXFA 526	6.46			
LXFL 529	9.86	23.07	31.85	25.9
MAXF MDA-MB-231	3.2	10.55	5.14	5.51
MAXF SK-BR-3	8.53	19.99	28.23	25.03
PAXF 1657	6.4	18.87	13.06	14.51
RXF SN12C	10.67	29.53	30.55	30.55
Geo Mean IC_{50}	6.98	14.91	12.33	10.66

3.2. Anti-cancer Activity of A. tinctoria

The 100 % ethanol extract of *A. tinctoria* exhibited the most potent *in vitro* anti-cancer activity against MDA-MB-231 cells (IC_{50}: 2.98 µg mL^{-1}, IC_{70}: 6.03 µg mL^{-1}) then on CAL-27 cells (IC_{50}: 3.75 µg mL^{-1}, IC_{70}: 5.59 µg mL^{-1}), whereas 100 % methanol extract of *A. tinctoria* showed more sensitivity towards CAL-27 cells (IC_{50}: 3.86 µg mL^{-1}, IC_{70}: 5.97 µg mL^{-1}) then on MDA-MB-231 cells (IC_{50}: 8.97 µg mL^{-1}, IC_{70}: 24.08 µg mL^{-1}) (Table 3). Both the 100 % ethanol and methanol extracts of *A. tinctoria* displayed dose-dependent decline in the cell survival of different tumor cells (Figure 1A and B), whereas, the 50 % ethanol and hot aqueous extract of *A. tinctoria* showed less anti-cancer activity (T/C: 29 % and 82 % respectively)

Table 3. The Minimum inhibitory concentration of the root extract of *Alkanna tinctoria* on a panel of tumor cell lines.

Cell line	*A. tinctoria* 100% ethanol extract (µg/mL)		*A. tinctoria* 100% methanol extract (µg/mL)	
	IC_{50}	IC_{70}	IC_{50}	IC_{70}
BXF RT112	4.94	7.95	6.63	11.18
CXF COLO 205	10.31	11.08	31.62	33.84
GXA MKN45	9.87	13.05	11.10	16.79
HNXF CAL-27	3.75	5.59	3.86	5.97
LXFA 526	5.99	9.80		
LXFL 529	8.32	15.12	16.21	29.18
MAXF MDA-MB-231	2.98	6.03	8.97	24.08
MAXF SK-BR-3	7.70	11.15	17.23	31.28
PAXF 1657	6.02	8.23	17.89	33.6

Figure 1. Dose response curve of anti-cancer efficacy of the root extract of *Alkanna tinctoria* on panel of tumor cell lines. A: *A. tinctoria* root extracted with 100 % ethanol); B: *A. tinctoria* root extracted with 100 % methanol. Y axis indicates the percentage of survival. T/C: Test/Control.

3.3. Anti-Cancer Activity of R. tinctorum

The 100 % methanol extract of *R. tinctorum* was sensitize CAL-27 cells (IC_{50}: 2.30 µg mL^{-1}, IC_{70}: 3.36 µg mL^{-1}) followed on MDA-MB-231 cells (IC_{50}: 5.14 µg mL^{-1}, IC_{70}: 12.99 µg mL^{-1}) whereas, the 50 % ethanol extract of *R. tinctorum* sensitize CAL-27 cells (IC_{50}: 2.64 µg mL^{-1}, IC_{70}: 4.04 µg mL^{-1}) followed on MDA-MB-231 cells (IC_{50}: 5.68 µg mL^{-1}, IC_{70}: 12.37 µg mL^{-1}) (Table 4). Similar to *A. tinctoria* extracts, the 100 % methanol and 50 % ethanol extracts of *R. tinctorum* also showed a dose-dependent decline in the survival of tumor cells (Figure 2A and 2B).

Table 4. The Minimum inhibitory concentration of the root extract of *Rubia tinctorum* on a panel of tumor cell lines.

Cell line	*R. tinctorum* 50% ethanol extract (µg/mL)		*R. tinctorum* 100% methanol extract (µg/mL)	
	IC_{50}	IC_{70}	IC_{50}	IC_{70}
BXF RT112	5.02	9.63	3.17	5.38
CXF COLO 205	22.13	33.01	18.94	26.61
GXA MKN45	9.46	13.30	6.74	9.82
HNXF CAL-27	2.64	4.04	2.3	3.36
LXFA 526				
LXFL 529	31.44	81.82	19.19	4.19
MAXF MDA-MB-231	5.68	12.37	5.14	12.99
MAXF SK-BR-3	26.25	41.92	26.35	40.89
PAXF 1657	13.02	31.37	15.56	26.55
RXF SN12C	33.58	69.34	28.53	56.37

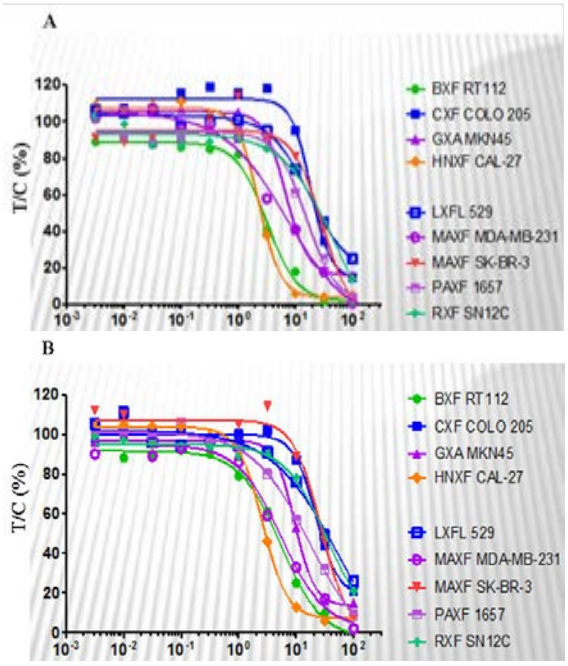

Figure 2. Dose response curve of anti-cancer efficacy of the root extract of *Rubia tinctorum* on a panel of tumor cell lines. A: *R. tinctorum* root extracted with 100 % methanol; B: *R. tinctorum* root extracted with 50 % ethanol. Y axis indicates the percentage of survival. T/C: Test/Control.

4. Discussion

It is well accepted that the plant constituents possess therapeutic and preventive activities against different cancer types. The Potential natural products have already contributed to 60 % of all anti-cancer drugs (Rates, 2001; Dorai and Aggarwal, 2004; Rabi and Bishayee, 2009). Various epidemiological and preclinical findings and the results of several early clinical studies convincingly argue for a definitive role of some selected dietary products in the treatment and prevention of cancers. Many of these agents target multiple signal transduction pathways, modulate cancer aneuploidy, tubulin binding, topoisomerases, and gene-specific targets, which vary widely depending on cancer origin (Pezzuto, 2008; Amin *et al*., 2009; Nobili *et al*., 2009). Optimizing the extraction

strategy to achieve a high yield of anti-cancer active principles is the paramount interest of cancer drug discovery researchers. Choosing an ideal solvent system and extraction stringency would pave the way for isolating a significant amount of active principles from natural resources (Zlotek *et al.,* 2016). In this study, different concentrations (50 % and 100 %) of ethanol, methanol and water (hot) were employed for the extraction. Both water and alcohols (ethanol and methanol) are well-known to extract polyphenolic compounds due to their high polar nature. *R. tinctorum* and *A. tinctoria* are well established for different biological activities such as antioxidant, anti-analgesic, anti-cancer etc. In particular, root extracts from *R. tinctorum* and *A. tinctoria* are reported for the presence of high amount of polyphenols alizarin and alkannin respectively (Tappeiner *et al*., 2014; Eszter Lajko *et al*., 2015). Unfortunately, *R. tinctorum* and *A. tinctoria* which are endemic in Jordan are not yet well-explored for any biological activities in particular, as an anti-cancer agent. To address this issue, we collected *R. tinctorum* and *A. tinctoria* root from local regions of Jordan and studied their anti-cancer activity in a panel of tumor cell lines (tongue, bladder, colon, gastric, lungs, breast, pancreas, and renal).

The extracts were tested at ten different concentrations in half-log dilution steps. Anti-tumor activity is expressed as absolute IC_{50} and IC_{70} values, calculated by non-linear regression analysis. Less than 5 µg mL^{-1} of 100 % methanol extract of *A. tinctoria* restricted the proliferation of MDA-MB-231 and CAL-27 cells significantly. In Jordan, the whole plant ethanolic extracts of *Alkanna strigosa* were previously tested for anti-cancer activity against MCF-7 cells, and it was reported that MCF-7 cells withstand the treatment and exhibit a 99 % survival (Abu-Dahab and Afifi, 2007). Interestingly, the plants belong to similar genus and endemic elicited difference in anti-cancer activities. This variation could be attributed to the difference in the presence of anti-cancer active principles. The presence of phenolic compounds such as alkannin, acetylalkannin, propionylalkannin, isobutylalkannin, angelylalkannin, β, β-dimethylacrylalkannin, isovaleryl-alkannin, α-methyl-n-butylalkannin, teracryl-alkannin, β – hydroxyisovalerylalkannin and naphtha-quinones in the root extract of *A. tinctoria* is well- reported (Assimopoulou *et al.,* 2006). Alkannin and angelylalkannin isolated from a 95 % ethanol root extract of *A. tinctoria* were shown to be effective anti-colon cancer agents (Tung *et al*., 2013). In contrast, this study found that both CAL-27 and MDA-MB-231 cells are more sensitive than colon cancer cells (COLO205). Specific anti-proliferative property of 100 % ethanol and methanol root extracts of *A. tinctoria* against CAL-27 and MDA-MB-231 cells is not clear. High polar organic solvents ethanol and methanol are widely used and recommended to extract potential polyphenolic compounds (Tomsone *et al*., 2012; Goncalves *et al*., 2015). Synergistic mixture of phenolic compounds with the presence of solvents could achieve inter and intra molecular interaction and novel stereochemistry (Freeman *et al*., 2010) and this may pave the way for specific anti-tumor potential towards CAL-27 and MDA-MB-231 cells. The chemical composition of 100 % ethanol and 100 % methanol extracts of *A. tinctoria* root should be studied in detail to address this issue further.

Mazzio *et al*. reported that the ethanolic root extract of *R. tinctorum* can restrict the growth of MDA-MB-231 cells significantly with an IC_{50} value of 20.5 µg mL^{-1} (Mazzio *et al.,* 2014). In the current study, it was found that both the 50 % ethanol (5.14 µg mL^{-1}) and the 100 % methanol (5.68 µg mL^{-1}) extracts of *R. tinctorum* reduced the growth of MDA-MB-231 cells with a less concentration than reported before. Noticeably, the 50 % ethanol extract of *R. tinctorum* showed a better anti-cancer activity than the 100 % methanol extract. Mixing water with alcohol could enhance the polarity further, thus, aqueous alcoholic extract could extract a high amount of phenolic compounds than absolute solvent extracts. Sultana *et al.* reported that aqueous alcoholic extraction can achieve a high yield of antioxidant phenolic compounds than absolute solvent extraction (Sultana *et al*., 2009). The root extract of *Rubia cordifolia* L contains a rich amount of the phenolic compound mollugin inhibiting the proliferation of HeLa cells by modulating TNF-α and NF-kB signaling (Zhe Wang *et al*., 2017). Roots of *Rubia yunnanensis* contain triterpenoid rubiarbonol G induced apoptosis and cell cycle arrest in HeLa cells (Zeng *et al*., 2017). *R. tinctorum* is known to accumulate a rich amount of phenolic compounds such as alizarin, purpurin etc. **(**Eszter Lajko *et al*., 2015**).** Hydroxyl group of phenolic compounds can intercalate with DNA and induce irreparable DNA damage and apoptosis. Water extraction can achieve a high yield of phenolic compounds compared to organic solvent due to its high polarity; however, it depends on the nature of plants and its parts. Naturally, the roots are the harder part of plant compared to the leaves and stem. The aqueous extract is widely considered to be safe for oral ingestion for the various ailments; however, the efficient extraction of active principles from plant parts depends on the vulnerability of cellular architecture of plant parts. In this study, it was found that the root extracts obtained from *A. tinctoria* and *R. tinctorum* using alcohol can sensitize the tumor cells more significantly than the aqueous extract. It clearly indicates that to extract the active principles from the roots of *A. tinctoria* and *R. tinctorum* requires organic solvents due to their harder nature, for which water may not be sufficient to destruct the cellular architecture to achieve a high yield of the phenolic compounds.

5. Conclusion

The results of the present investigation revealed that the root extracts of *R. tinctorum* were found to be more potent anti-cancer agents compared to the *A. tinctoria* root extracts. It is recommended that the chemical composition of the *R. tinctorum* root extracts should be studied in detail. Studies on the efficacy of root extracts on apoptosis (intrinsic and extrinsic) and on cell survival signaling molecules should be done to find out molecular mechanism. Further research is needed to assess the anti-cancer effect of root extracts' derived compounds *in vivo* model and to develop cancer drug formulation from *R. tinctorum.*

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Phytochemical Screening: Antioxidant and Antibacterial Activities of *Verbena supina* L. Aqueous, Hexane and Methanol Extracts

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Abstract

This study evaluates the phytochemical constituents of *Verbena supina* L aqueous, hexane and methanol extracts as well as their antioxidant and antibacterial activities. *Verbena supina* L extracts are studied here in order to support their common use in the traditional medicine. Antioxidant activities were assessed employing 2,2-diphenyl-1-picrylhydrazyl (DPPH) free-radical scavenging method, and the antibacterial activities were tested using broth microdilution method against three bacteria; *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 25922, and *Pseudomonas aeruginosa* ATCC 27853. The methanolic extract showed the highest antioxidant activity with IC_{50}value of 6.7±1.37 µg/mL; it was followed by the aqueous extract (IC_{50}= 9.9±1.20 µg/ml), and the hexane extract (IC_{50}=19.9±1.05 µg/mL). In addition, the *V. supina* methanolic extract showed the the strongest antibacterial activity against *S. aureus* and *E. coli* strains with a MIC value of 0.25 mg/mL and 1.75 mg/mL, respectively.

Keywords: *Verbena supina*, Antimicrobial, Antioxidant, Phytochemicals.

1. Introduction

Verbena supina L., commonly known as trailing vervain, belongs to the Verbenaceae family. It is frequently grown as small clumps, but seldom occurs as insufficient quantity to be considered a weed (Cunningham *et al.,* 2011). The species is native to the Mediterranean region. Its habitat ranges from southern and eastern Europe, northern and eastern tropical Africa, to the Middle East, Pakistan, Malaysia and Australia (Munir, 2002). The plant species of *Verbena* are used in numerous cases in traditional medicine as anti-depressants, anti-jaundice and anti-inflammatory medications, and also as diuretics and expectorants, as well as anti-influenza medications, (Al-Amier *et al.,* 2005; Wichtl, 2004). In fact, the most common species used in traditional medicine is *Verbena officinalis* (Mengiste *et al.,* 2015; Deepak and Handa, 2000; Casanova *et al.,* 2008; Tang and Eisenbrand, 1992); however, other sub species of *Verbena* are possibly interchangeable.

The phytochemical investigation and the isolated compounds of *Verbena* have shown the presence of polyphenols, flavonoids, terpenoids, glycosides, and saponins (Soares *et al.,* 2016; Abebe *et al.,* 2017; Verma and Siddiqui, 2011; Kawashty and El-Garf, 2000). Moreover, they have shown multiple biological activities such as anti-tumor (Kou *et al.,* 2013), antioxidant (Abebe *et al.,* 2017), analgesic (Abdelshafeek *et al.,* 2010) and anti-inflammatory activities (Calvo, 2006), in addition to their antidepressant (Jawaid *et al.,* 2015), antinociceptive (Braga *et al.,* 2012), neuroprotective effects (Lai *et al.,* 2006). They also are effective in enhancing the activity of nerve growth factors (Li *et al.,* 2003). Furthermore, recent research has demonstrated that the plant has anticonvulsant, anxiolytic, and sedative activities (Khan *et al.,* 2016). Such biological activity reports explain its common use in folk medicine.

The scientific literature on *Verbena supina* L., shows that its essential oil exhibits antimicrobial, antifungal and phytotoxic activities. Moreover, its flavonoids and total polyphenol content were previously studied (Al-Amier *et al.,* 2005; Kawashty and El-Garf, 2000; Dallali *et al.,* 2014), and in order to further develop the phytochemical and biological activity investigation of this plant, the aqueous, methanol and *n*-hexane extracts of *V. supina* were subjected in the current study to phytochemical screening followed by antioxidant and antibacterial activity investigations.

2. Materials and Methods

2.1. Plant Materials

All of the *V. supina* plant samples were collected from the mountains of Hebron region of Palestine during July, 2016. The plant has been botanically identified by pharmacognosist Dr. Nidal Jaradat from the Pharmacy Department at An-Najah National University. A voucher specimen Pharm-PCT-2608 has been retained in the

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herbarium of the Laboratory of Pharmacognosy. The plant was washed well several times with distilled water, and was then dried in the shade for four weeks at room temperature. After drying, the leaves were grounded into fine powder using a blender. The powder was then kept in airtight containers with proper labeling for future use.

2.2. Chemicals and Equipment

Methanol, *n*-hexane, Millon's reagent, Benedict's reagent, Sodium hydroxide, magnesium ribbon, and acetic acid were obtained from Lobachemie (India). While Trolox ((s)-(-)-6 hydroxy-2,5,7,8-tetramethychroman-2-carboxylic acid), (DPPH) 2,2-Diphenyl-1-picrylhydrazyl, chloroform, and hydrochloric acid were obtained from Sigma-Aldrich, Germany. Ninhydrin solution, Molish's reagent, sulfuric acid, and Iodine solution were obtained from Alfa-Aesar, England. $FeCl_3$ from Riedeldehan, Germany. Nutrient broth from Himedia (India). Dimethyl sulfoxide (DMSO) and antibiotics from OXOID, England. Moreover, the instruments used in the current study include: Spectrophotometer (Jenway 7135, England), Freeze dryer (Mill rock technology, model BT85, Danfoss, China), Shaker device (Memmert Shaking Incubator, Germany), Rotary evaporator (Heidolph VV2000, Germany), Grinder (Moulinex model, Uno. China), Filter papers (Whatman no.1, USA and Machrery-Nagel, MN 617), Micro-broth plate (Greiner bioone, North America), Micro-pipettes (Finnpipette, Finland), Syringe filter 0.45 µm pore size (Microlab, China), and incubator (Nuve, Turkey).

2.3. Preparation of Plant Extracts

The powdered plant material was extracted in hexane and methanol separately at a 10 % (w/v) concentration (25 g powder in 250 mL *n*-hexane or methanol) using Soxhlet apparatus for seven hours. The extracts were evaporated over a water bath at 30-40ºC, and were then kept in a refrigerator at 2-8 ºC until further use. Also, some powdered plant material was extracted in distilled water at a 10 % (w/v) concentration (25 g powder in 250 mL distilled water). The mixture was heated and stirred in a hot plate at 30º-40ºC for twenty minutes; then the extract was filtered through filter paper. The filtrate was used for the phytochemical analysis.

2.4. Antioxidant Activity

The free radical scavenging activity of the *V. supina* extracts and standard were measured according to the procedure described earlier (Jaradat *et al.,* 2015). DPPH radical solution at a concentration of 0.002 % w/v was mixed with methanol and the prepared concentrations (1, 2, 3, 5, 7, 10, 20, 30, 40, 50, 80, 100 µg/mL) from three plant extracts of 1mg/mL in methanol and standard Trolox in a ratio of 1:1:1, respectively. The solutions were left in dark incubation for thirty minutes at room temperature. Absorbance readings were recorded at 517 nm. The percentage of inhibition of DPPH activity (I %) was calculated using the following equation (Jaradat *et al.,* 2015):

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

where: A_{blank} is the absorbance of the control reaction (containing all reagents except the test compound), A_{sample} is the absorbance value of the extract.

The antioxidant half maximal inhibitory concentration (IC_{50}) for the *V. supina* plant and Trolox standard at different concentration were plotted and tabulated, and the IC_{50} for each one of them was calculated using the BioDataFit fitting program.

2.5. Qualitative Phytochemical Analysis

Preliminary qualitative phytochemical screening of primary and secondary metabolic compounds such as proteins, starch, phenols, cardiac glycosides, saponin glycosides, flavonoids, alkaloids, steroids, volatile oils, and tannins were carried out according to the standard common phytochemical methods described by Trease and Evans (1983), and Harborne (1998) for *V. supina* entire plant.

2.5.1. Antibacterial Evaluation

Antibacterial activity of aqueous, *n*-hexane and methanolic extracts of *V. supina* were tested against four bacteria, namely (*Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, and *Acinetobacter baumannii* ATCC BAA 2802) by broth microdilution method following the Clinical Laboratory Standards Institute (CLSI) recommendations (Forbes *et al.*, 2002).

An aqueous extract solution of 100 mg/mL was prepared in sterile distilled water. The *n*-hexane and methanol extracts of 178 mg/mL were prepared with 100 % Dimethyl sulfoxide (DMSO), syringe filter 0.45 µm pore size was used to sterilize the solutions. Then, the aqueous, hexane and methanol extract solutions were serially diluted 2-folds with nutrient broth in a micro-broth plate. Well number eleven was a negative control of bacterial growth, while well number twelve was for positive control of bacterial growth and contained nutrient broth. ten concentrations of aqueous, and organic extract solutions were obtained from 0.098 to 50 mg/mL and 0.174 to 89 mg/mL, respectively. Dimethyl sulfoxide (DMSO) was also serially diluted 2-fold with nutrient broth and was prepared with concentrations from 0.098 % to 50 %. The final bacterial concentration in each well (except negative control) was adjusted to 5×10^5 CFU/mL by transferring 50 µL of the 0.5 McFarland standard equivalent suspension to 10 mL of broth, then the plates were covered and incubated at 35° C for eighteen hours. Each of the bacterial isolates were examined in duplicate. Minimal inhibitory concentration (MIC) was considered when the lowest concentration of the plant extract did not allow any visible bacterial growth in the test broth.

3. Results and Discussion

3.1. Phytochemical Screening

The phytochemical characteristics of *V. supina* plant are summarized in Table 1.

It shows that proteins, tannins, flavonoids, glycosides and terpenoids were found in *V. supina* plant, whereas carbohydrates, saponins and alkaloids were absent.

Table 1. Phytochemical constituents of *V. supina*.

	Aqueous extract	Methanolic extract	*n*-hexane extract
Proteins	+	+	+
Carbohydrate	–	–	–
Phenols/Tannins	+	+	+
Flavonoids	+	+	+
Saponins	–	–	–
Glycosides	+	+	+
Steroids	-	-	-
Terpenoids	+	+	+
Alkaloids	–	–	–

3.2. Antioxidant Activity

The methanolic extract of *V. supina* has the highest free radical scavenging activity followed by the aqueous, and hexane extracts. The IC_{50} of methanol, aqueous and hexane extracts were 6.7±1.37 µg/mL, 9.9±1.20 µg/mL and 19.9±1.05 µg/mL respectively. The IC_{50} of Trolox standard reference was of 4.8±1.39 µg/mL. Detailed results are shown in Table 2

Table 2. Inhibition activity for Trolox Standard and *V. supina* entire plant.

Concentration of methanol extract DPPH\ methanol	% of inhibition by Trolox	% of inhibition by *V. supina* methanol extract	% of inhibition by *V. supina* *n*-hexane extract	% of inhibition by *V. supina* aqueous extract
1µg\mL	21.9±1.20	33.92±0.98	3.35±0.15	19.95±0.66
2 µg\mL	38.15±1.44	43.85±1.22	11.11±0.87	21.69±1.35
3 µg\mL	43.19±1.35	50.35±1.38	13.16±0.87	22.84±0.98
5 µg\mL	63.19±1.12	56.97±1.78	17.53±0.94	34.97±1.11
7 µg\mL	68.78±1.45	66.76±1.31	19.49±0.99	46.92±1.12
10 µg\mL	77.9±1.22	70.55±1.65	25.36±1.11	58.78±1.2
20 µg\mL	79.15±1.35	70.59±1.55	31.23±1.12	64.25±1.13
30 µg\mL	81.12±1.74	72.46±1.44	36.13±1.36	74.85±1.27
40 µg\mL	81.9±1.25	73.69±1.27	37.05±1.41	82.95±1.55
50 µg\mL	83.95±1.77	78.45±1.34	36.97±1.25	86.98±1.35
80 µg\mL	84.85±1.55	83.53±1.35	46.18±1.25	92.2±1.25
100 µg\mL	90.7±1.35	93.76±1.25	53.4±1.34	95.75±1.47
IC_{50}	4.8±1.39	6.7±1.37	19.9±1.05	9.9±1.20

3.3. Antibacterial Activity

The MIC values of aqueous and organic extracts of *V. supina* against examined bacterial isolates are shown in Table 3.

Table 3. Antibacterial activity of aqueous, methanol and *n*-hexane extracts of *V. supina* plant.

Bacterial strains	MIC value (mg/mL)		
	aqueous extract	methanol extracts	*n*-hexane extract
S. aureus	20	0.25	1.5
E. coli	20	1.75	3.75
P. aeruginosa	25	6.5	2.5
A. baumannii	40	6.5	1.5

The aqueous extract of *V. supina* showed antibacterial activity MIC = 20 mg/mL against *S. aureus* reference isolate, which is a Gram-positive bacterium. In addition, the *V. supina* aqueous extract was found to exhibit similar antimicrobial activities MIC = 20 mg/mL against *E. coli* Gram-negative bacterial isolates of the present study. The MIC value of the aqueous extract against *P. aeruginosa* was equal to 25 mg/mL. The Aqueous extract of *V. supina* showed a lower level of activity MIC= 40 mg/mL against *A. baumannii* Gram-negative. Obviously, the antimicrobial activity of methanol and hexane extracts of *V. supina* were stronger than the aqueous extract. Among the examined isolates, the methanolic extract showed the strongest activity against *S. aureus* and *E. coli* MIC = 0.25 mg/mL and 1.75 mg/mL respectively. It was moderately followed by the *n*-hexane extract against *S. aureus* and *A. baumannii* MIC = 1.5 mg/mL. as for the methanolic extract against *P. aeruginosa* and *A. baumannii*, MIC = 6.5 mg/mL; on the other hand, MIC of the hexane extract against *P. aeruginosa* and *A. baumannii* was 2.5 mg/mL and 1.5 mg/mL respectively. A lower level of inhibition was examined between Gram negative and Gram-positive isolates. However, the activity of both methanol and hexane extracts against Gram-negative bacteria was much higher than that of the aqueous extract. Methanolic and hexane extract activities against the bacterial isolates could be related to the outer membrane in Gram-negative bacteria but not Gram-positive bacteria.

4. Conclusion

In conclusion, the current study showed that the methanolic extracts of *V. supina* have a high content of phenolic compounds and show a high antioxidant activity, therefore, they can be used to treat several diseases that are on the rise. in which there is an increase in free radical production. Also, the *V. supina* methanolic and hexane extracts, possess significant antibacterial activity against the Gram-positive *S. aureus* and strong antibacterial activity against the Gram-negative bacterial *E. coli*. The current study also shows that the antibacterial activity of the *V. supina* methanolic and hexane extracts was stronger than that of the aqueous extract. However, further studies are still needed to identify which phytogenic phenols are responsible for the antioxidant activity of the entire plant, and to assess the way in which the phytogenic substances contribute to this activity.

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The Efficacy of Photosensitizers on Mycelium Growth, Mycotoxin and Enzyme Activity of *Alternaria* spp.

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Abstract

Vegetables are subjected to heavy yield losses in quality and quantity as a result of various diseases caused by dematiaceous fungi. However, the use of fungicides is hazardous to humans, animals, and the environment. In this study, emphasis is given to other methods of disease control through the employment of photodynamic treatments such as photosensitizers which are considered safer, more economical, and eco-friendly. The objective of the current study was to evaluate the possibility of using photodynamic inactivation against *Alternaria alternata*, the causal agent of tomato blight. Samples of tomato, squash, pepper and cucumber were collected from different fields, and the isolated fungi were identified morphologically and molecularly. The inhibitory doses of the photosensitizer regarding the growth of *A. alternata* were studied. The results showed that 100 µg/mL of toluidine blue-O (TBO) was effective inhibiting *Alternaria* spp. with a significance variation among different species of *Alternaria*. The *A. alternata* isolated from tomato was more resistant to TBO and less sensitive to light compared to other isolates. The effect of the photosensitizer on cellulase and pectinase as well as the production of alternariol mycotoxin were studied. The results showed that the photosensitizer was inhibitory for enzyme activity and the alternariol production especially in the presence of light. In conclusion, photosensitizers can be used for treating plant pathogenic fungi such as *Alternaria* spp.

KeyWords: *Alternaria* spp., Photosensitizer, Cellulase, Pectinase, Mycotoxin.

1. Introduction

Vegetables constitute the most important and inexpensive component of a balanced diet due to their high nutritional values indispensable for the body. Cucurbitaceae, Brassicaceae and Solanaceae families are considered important vegetables due to their nutritional and economical values, however, various diseases caused by dematiaceous fungi may lead to heavy yield losses in the quantity and quality. Dematiaceous fungi are characterized by a dark cellular pigment, resulting from a melanization process. Jacobson (2000) and Nosanchuck and Casadevall (2003) linked melanin with virulence, resistance, and susceptibility in plants.

Different *Alternaria* spp. were reported to be associated with different angiospermic families, but *Alternaria alternata* -a dematacious fungus- cause early blight diseases to Cucurbitaceae, Brassicaceae and Solanaceae families (Neeraj and Verma, 2010). Furthermore, different species of *Alternaria* had family-pathogenic infection specificity, for example, *A. tenuissima* and *A. cucumerina* on cucurbitaceous; *A. brassicae*, *A. brassicicola* and *A. raphani* on brassicaceous and *A. solani*, *A. longipes* and *A. crassa* on solanaceous plants (Neeraj and Verma, 2010).

There are several studies (Abada *et al*., 2008; Agamy *et al*., 2013; Bhatti *et al*., 2002; Metz, 2017) which investigated the controlling procedures for the early blight of these vegetables. One of the most commonly applied methods is the use of fungicides, but these compounds cause serious health hazards to human beings and cause environmental pollution. Hence, nowadays more emphasis is given to other methods of disease control. One of these methods is the employment of photodynamic treatments by using photosensitizers which are considered safer, more economical, and eco-friendly.

Several compounds when irradiated (photoactive compounds or photosensitizers) can cause toxic reactions in living cells (Matos and Ricardo, 2003). The importance of these compounds may increase, if they can react with different microbes that affect human or animal health, and crops. Polyetilenes, thiophenes, coumarins, furano-cocumarins, furanocromones, β-carbolines and other alkaloids and complex quinines are the main classes of plant photosensitizers (Arnason *et al*., 1992). By absorbing light, photosensitizers can trigger chemical modifications

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of a substrate or target. Photosensitizers are considered among the new environmentally safe and harmless pesticides to non-target microorganisms for crop protection.

The first utilization of light effects was for the control of insects (Robinson, 1983). At present, photosensitizers are promising compounds used to control insects, nematodes, weeds, algae, viruses, bacteria, yeast and fungi as well as tumor cells (Berenbaum, 1987; Wainwright, 1998; Hamblin and Hasan, 2004). A large number of synthetic and natural compounds for antimicrobial photodynamic therapy has been developed (Jori, 2006). Acridine hydrochloride used for the photoinactivation of microorganisms was the first to be described (Raab, 1900). The phenothiazine dyes as methylene blue and toluidine blue showed promising results against bacteria and fungi (Wainwright, 1998). The photosensitizers with highly conjugated molecules such as porphyrins and phthalocyanines possess improved optical properties (Sternberg and Dolphin, 1998). Phthalocyanines, which are characterized by far red wavelength absorption (>670 nm), long triplet life time (ms), and high quantum yields of singlet oxygen generation (>0.2), have been studied as drugs in microbial photodynamic inactivation (Jori and Tonlorenzi, 1999).

The photocatalytic technology is based on the interaction of light with semi-conductor particles to produce highly Reactive Oxygen Species (ROS) which destroy microbial cells (Biel, 2010). Thus, photochemically-active compounds in catalytic amounts (Aromatic Photosensitizers or APS) may be considered (Hamblin and Hasan, 2004). The elimination of microorganisms using APS is known as antimicrobial Photo-Dynamic Inactivation (PDI) (Maisch, 2007), and it uses visible light to activate the photosensitizers. The absorption of a photon by a photosensitizer leads to the production of ROS according to two pathways: (i) radicals formation, issued from electron transfer from the photosensitizer to molecules in its direct environment (Type I photo-oxidation reaction). In the presence of air, the most often produced ROS is thus superoxide radical-anion, $O_2^{\bullet -}$, (ii) energy transfer from the triplet excited state of photosensitizers to the ground state of oxygen, generating singlet oxygen, $^1O_2$, superoxide and radicals (Type II photo-oxidation reaction). These reactive species can oxidize the surrounding bioorganic molecules, such as proteins, nucleic acids, lipids, leading to cell death.

Toluidine blue (TBO) as antimicrobial photodynamic or photosensitizing agent is a promising method that can be used to control the phytopathogens. This method has been tried successfully under in vitro conditions against *Metarhizium anisopliae* and *Aspergillus nidulans* under different incubations of light conditions (Gonzales *et al*., 2010).

The objectives of the current study were: (i) to evaluate the impact of the photosensitizer in the presence of light on controlling the phytopathogenic *Alternaria* spp., and (ii) to determine the efficacy of photosensitizer on reducing secreted toxins, and (iii) to determine the efficacy of photosensitizer on inhibition of the hydrolytic degrading enzymes of isolated fungi in order to evaluate the possibility of using photozensitizer in plant protection against phytopathogenic fungi, and to reduce the use of hazardous chemical pesticides.

2. Materials and Methods

2.1. Collection, Isolation, Purification, and Preservation of Fungal Isolates

The samples for this study were collected in 2012 from naturally-infected vegetables including cucumbers (*Cucumis sativus* L.), squash (*Cucurbita pepo* L.), tomatoes (*Solanum lycopersicum* L.*)*, and pepper (*Capsicum annuum* L.) grown in different fields in Almadinah Almunawwarah region. The pathogens were isolated from different parts of the plant, such as the fruits, leaves, and stems which show symptoms of infection (Table 1).

For isolation, a small piece was removed from the lesion and healthy tissue of diseased plants and the surface was sterilized by 1 % sodium hypochlorite solution, then washed with sterile distilled water for one minute. After that, the specimen was air-dried under the laminar flow on sterilized filter paper, transferred to Potato Dextrose Agar plates (PDA) (formula/Liter; potato extract 4.0g, dextrose 20.0g, agar 15.0g; 4.0g of potato extract equivalent to 200g of infusion from potatoes (Oxoid LDT., Basingstoke, Hampshire, England), and incubated at 28º C±2 for one week.

A pure culture of each fungus was obtained by hyphal tipping, and the isolates were incubated onto PDA plates. For preservation, about ten agar plugs containing young and actively growing mycelia were taken from the margins of the colony using sterile 4-mm diameter cork-borer and were transferred into glass vials filled with 70 ml sterile distilled water and 30 ml glycerol and kept at -20° C.

Table 1. Information concerning host, locations and identity of the isolates collected from different fields at Almadinah Almunawwarah..

Isolate number	Isolate ID	Host	Field number	Location (GPS coordinate)		Organism	Gene bank accession number
				Coordinate 1	Coordinate 2		
2	AjKSA12-01	Tomato	F1	N24.620	E36.92	*A. japonica*	KF944473
5	PodKSA12-01	Tomato	F1	N24.620	E36.92	*Podospora* sp.	KF944474
6	AaKSA12-01	Tomato	F2	N24.709	E40.13	*A. alternata*	KF944475
7	ChaetKSA12-	Tomato	F1	N24.620	E36.92	*Chaetomium*	KF944476
8	AaKSA12-02	Tomato	F1	N24.620	E36.92	*A. alternata*	KF944477
9	TharKSA12-01	Tomato	F1	N24.620	E36.92	*Thielavia*	KF944478
10	PtKSA12-01	Tomato	F1	N24.620	E36.92	*Phoma tropica*	KF944479
11	AaKSA12-03	Tomato	F2	N24.709	E40.13	*A. alternata*	KF944480
12	AaKSA12-04	Tomato	F1	N24.620	E36.92	*A. alternata*	KF944481
13	AaKSA12-05	Tomato	F2	N24.709	E40.13	*A. alternata*	KF944482
14	AaKSA12-06	Tomato	F2	N24.709	E40.13	*A. alternata*	KF944483
16	AaKSA120-07	Squash	F3	N24.375	E39.54	*A. alternata*	KF944484
17	AaKSA12-08	Squash	F3	N24.375	E39.54	*A. alternata*	KF944485
18	AaKSA12-09	Squash	F3	N24.375	E39.54	*A. alternata*	KF944486
20	AaKSA12-10	Squash	F3	N24.375	E39.54	*A. alternata*	KF944487
21	AaKSA12-11	Squash	F3	N24.375	E39.54	*A. alternata*	KF944488
22	AaKSA12-12	Squash	F3	N24.375	E39.54	*A. alternata*	KF944489
23	AcKSA12-01	Pepper	F4	N24.515	E39.32	*A. compacta*	KF944490
24	AaKSA12-13	Pepper	F4	N24.515	E39.32	*A. alternata*	KF944491
25	ApKSA12-01	Pepper	F4	N24.515	E39.32	*A. porri*	KF944492
26	AaKSA12-14	Pepper	F4	N24.515	E39.32	*A. alternata*	KF944493

2.2. Identification of Fungal Isolates

2.2.1. Morphological Identification

Pure cultures were grown for seven days and were examined microscopically (Light Microscopic - LEICA DME) to view the morphological characters of the different fungi. Slide cultures were prepared for each isolate. They were photographed, and identified via identification key for the imperfect fungi of Barnett (1960), Barnett and Hunter (2003) and Ellis (1976).

2.2.2. Molecular Confirmation

A total of thirty isolates were sequenced in this study. The morphologically identified isolates were sub-cultured on PDA, and were sent for molecular identification to Fragment Analysis & DNA Sequencing Services (FADSS), Okanagan, British Columbia, Canada. Internal transcribed spacer region ITS4 (TCCTCCGCTTATTGATATGC) and ITS5 (GGAAG TAAAAGTCGTAACAAGG) were used for sequencing (White *et al*., 1990). DNA extraction, PCR's and sequencing using the ABI sequencer system were conducted on each isolate. Sequences were received as text files, and were edited via BioEdit version 5.0.6 (Hall, 2007) and BLASTn at the Gen Bank nucleotide database (http://www.ncbi.nlm.nih.gov/). Both, sequences of the thirty isolates and the reference isolates from the GenBank were aligned and a tree was executed using the software MEGA 5.1 (Tamura *et al*., 2011). The Maximum Likelihood method based on the Tamura-Nei model was used (Tamura and Nei, 1993). The bootstrap consensus tree was inferred from 1000 replicates (Felsenstein, 1985). Branches corresponding to partitions reproduced in less than 50 % bootstrap replicates were collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) was shown next to the branches (Felsenstein, 1985).

2.2.3. Fungal Isolates Used

Alternaria spp. were selected for studying the effect of the photosensitizer. Four species were chosen: *A. alternata* (AaKSA12-05 and AaKSA12-12), *A. compacta (*AcKSA 12-01), *A. porri (*ApKSA12-01), and *A. japonica* (AjKSA 12-01). *A. alternata* isolates were chosen because they were genetically different when they were molecularly confirmed.

2.3. Radiation and Photosensitization

2.3.1. White light lamp

The lamp (Lanzini Illuminazione, Italy) with a fluence rate of 400W/m^2 was used.

2.3.2. Photosensitizer

Toluidine blue O (TBO) (Sigma, UK) photosensitizer was used in this study. A 1000 µg/mL solution of TBO was used as stock solution in saline, and was kept at 2-4 C° in the dark. Different concentrations ranging from 0-100 µg/mL (0, 12.5, 25, 50, 100 µg/mL) were used for further experiments.

2.3.3. Linear Growth

Toluidine blue O (TBO) was prepared in different concentrations (125, 250, 500, and 1000 µg/mL). The medium was prepared in 500 mL flasks containing 270 mL sterilized Czapek Dox Agar (CZ) medium (formula/Liter; sucrose 30.0 g, $NaNO_3$ 2.0 g, K_2HPO_4 1.0 g, Mg_2SO_4 0.5

g, KCl 0.5 g, $FeSO_4$ 0.01 g, and agar 15.0 g) and 30 mL of TBO from each concentration. The control contained sterile distilled water instead of TBO. *Alternaria* spp. were grown on PDA from five to seven days depending on the species. A 10 mm diameter agar plug was transferred from PDA containing the fungal culture for each of the *Alternaria* spp. to a CZ plate that was previously treated with TBO. The culture of each species was divided into two groups: group 1 (no light) where the cultures were kept in the dark for sixty minutes, and group 2 (with light) where the cultures were exposed to fluent rate of 400 W/m^2 white light delivered for sixty minutes. After pre-incubated, both groups were incubated at 28°C in the dark. Colony diameters (in mm) were measured every forty-eight hours, until the mycelium filled the agar plate.

2.4. Production and Assay of Mycotoxin

In a preliminary experiment it was found that *A. alternata* was the only species that produces mycotoxins in a considerable amount. Therefore, the isolates AaKSA12-05 and AaKSA12-12 were selected for this purpose. For the mycotoxin production, Czapek Dox medium modified after Gatenbeck and Hermodsson (1965) was used (formula/Liter: 0.06 g, NH_4Cl, 0.25 g, $NaNO_3$, 1 g, KH_2PO_4, 0.5 g, $MgSO_4$ x 7 H_2O, 0.25 g, NaCl, 0.25 g, KCl, 0.01 g, $FeSO_4$ x 7 H_2O, 0.01 g, $ZnSO_4$ x 7 H_2O, 1 g, yeast extract, 30 g glucose (glucose is added separately after autoclaving). The pH was adjusted to 5.5. The inoculums were prepared as stated before in item 5.2. The alternariol was extracted from the medium with a mixture of acetonitrile and 4 % KCl in water (9:1 v/v, 40 mL) for thirty minutes, followed by the addition of 1 N HCl (8 mL). Alternariol was measured quantitatively by the mycotoxin analyzer (ROS-M Reader, Charm) using quantitative test kit (Burkin and Kononenko, 2011). The kit involves a quantitative lateral flow immunoassay with a range of sensitivity of 0 to 150 ppb and a limit of detection of 1 ppb.

2.5. Production of Pectinolytic and Cellulolytic Enzymes

Spore suspension of isolated AaKSA12-05 and AaKSA12-12 were prepared in 60 mL sterile distilled water flask. Then, 5 mL of the suspension were added to 5 ml of the different concentration of TBO (25, 50, 100 and 200 µg /mL). The control contained sterile distilled water instead of TBO. One group was kept in the dark for 60 minutes, and the other group was exposed to a fluent rate of 400W/m^2 white light delivered for sixty minutes. Ten milliliters of treated inoculums were grown on Czapek Dox broth (CDB) where the carbon source was substituted with one percent carboxy methyl cellulose (as substrate for cellulolytic enzymes), or one percent pectin (as substrate for pectinolytic enzymes). Carboxy methyl cellulose (CMC) medium consisted of (g/L): 7.5 g CMC, 7.5 g sucrose, 2 g $NaNO_3$, 1 g KH_2PO_4, 0.5 g $MgSO_4$, 0.5 g KCL, 0.01 g $FeSO_4$ g. Pectin medium has the same composition, but pectin replaced CMC. They were placed in incubator at 28°C in the dark for ten days. Then the treated inoculums were filtered for enzymes assay.

The cellulase and pectinase (polygalacturnase) activity were measured using the method of Bindo *et al*. (2005). The reaction mixture was prepared as follows ; 0.5 mL of 1 % substrate in phosphate citrate buffer (0.05 M, pH 5.2), 0.5 ml enzyme solution and 1 mL distilled water. They were placed in a shaking water bath at 50 °C for 10 minutes. Then three ml dinitrosalicylic acid reagent was added to stop the reaction. The tubes were heated in a boiling water bath for 5 minutes. After cooling, the tubes were centrifuged, and the absorbance was measured spectrophotometerically (APLE spectrophotometer PD-303UV) at 575 nm. One unit of enzyme activity (U) was defined as 1 µmol of reducing sugar released per minute.

2.6. Statistical Analysis

Analysis of variance (ANOVA) was performed using the PROC ANOVA in Statistical Analysis System (SAS 9.3; SAS Institute Inc., Cary, NC). Differences in means were compared using Fisher's least significant difference (LSD) test ($\alpha = 0.05$). Non parametric analysis was performed using MINITAB16 software (Minitab, State College, PA).

3. Results

3.1. Identification of Fungal Isolates

3.1.1. Morphological and Microscopical Characteristics

A total of thirty fungal isolates were collected from tomato, squash and pepper. Based on morphological characters, the isolates were related to six genera, namely: *Alternaria*, *Phoma*, *Chaetomium*, *Thielavia*, *Podospora* and *Trichoderma*. The most dominant genus was *Alternaria* which was represented by *A. alternat*a (n=23), *A. compacta* (n=1), *A. porri* (n=1) and *A. japonica* (n=1). As for the other genera, each was represented by one species namely: *Phoma tropica*, *Chaetomium* sp., *Thielavia arenaria*, *Podospora* sp. and *Trichoderma* sp.

3.1.2. Molecular Confirmation

The thirty isolates were sent for molecular identification and conformation. The ITS4/ITS5 gene region was amplified in the thirty isolates, but due to bad sequencing only twenty-one isolate were included in the dendogram (Figure 1). In the dendogram, eight species of the samples were shown clearly and included *Alternaria alternata*, *A. compacta*, *A. porri*, *A. japonica*, *Phoma tropica*, *Thielavia arenaria*, *Podospora* sp., and *Chaetomium* sp. (Figure 1). As shown in the dendogram, the *A. alternata* isolates recovered from the different hosts are distributed in different clusters indicating a genetic variation among the isolates. For example, *A. alternata* isolates number seventeen and twenty-two are found in cluster A, while isolates six and twenty-four are found in cluster B. Moreover, the *A. alternata* isolates within cluster A are found in different sub-clusters (Figure 1).

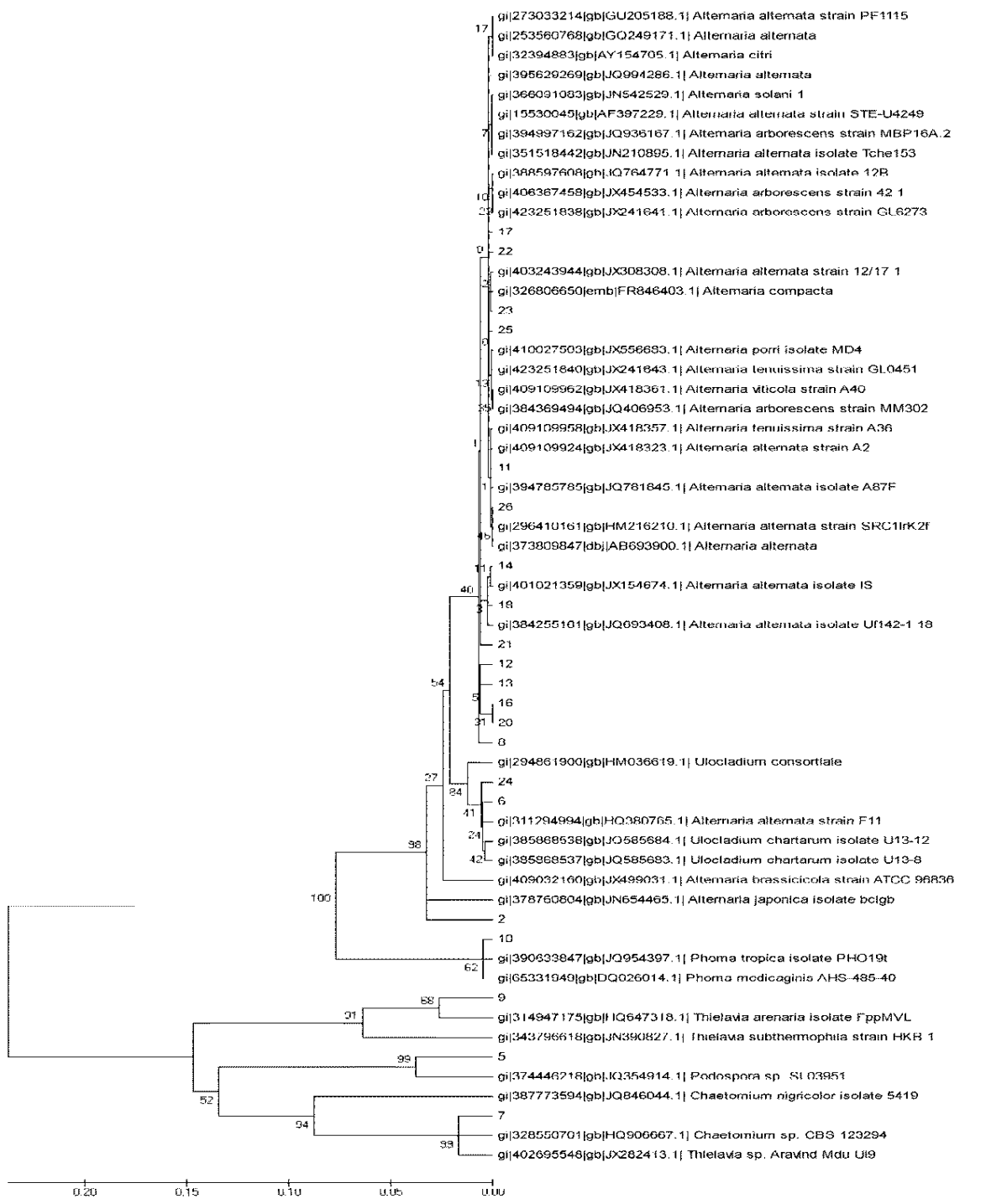

Figure 1. Dendogram of the 21 fungal isolates amplified using the ITS4/5 gene region. The dendogram was executed by MEGA 5 (Tamura *et al*., 2011) with 1000 bootstraps.

3.1.3. Linear Growth

Due to the dominance of *Alternaria* species in the isolated samples, the present work focused on the four *Alternaria* species, of which *A. alternata* was represented by two isolates. Among the five isolates of *Alternaria* spp. investigated in this study, *A. alternata* isolated from tomato had the highest mycelium growth regardless of the presence or absence of light compared to the other isolates (Table 2, Figure 2).

There were significant differences among the isolates (Table 3). Generally, the linear growth rate decreased as the concentration of the toluidine blue-O (TBO) increased with significant difference for the light, isolate, and concentration factors (Table 2, 3). Moreover, isolate-TBO concentrations' interaction showed highly significant differences (Table 2, 3, Figure 2).

Table 2. Linear growth (mm) of *Alternaria* spp. isolated from different hosts, treated with Toluidene Blue-O, and subjected to dark and light for 60 minutes [ab].

Isolate	Host	TBO (µg/ml) (Mean±se)									
		Dark					Light				
		0	12.5	25	50	100	0	12.5	25	50	100
Linear growth (mm)											
A.alternata	Tomato	66.84±3.45	62.00±2.18	55.17±1.54	53.38±1.28	49.78±0.65	57.33±1.70	54.26±1.84	49.25±1.50	47.57±1.14	42.15±0.46
A. alternata	Squash	61.03±2.56	55.73±2.78	46.08±3.11	43.89±2.59	40.26±1.60	53.90±1.32	47.42±1.27	39.58±2.21	34.18±1.07	30.96±1.18
A.compacta	Pepper	66.47±3.58	61.15±2.04	54.38±1.42	52.76±1.15	48.13±1.02	56.50±1.54	53.03±1.63	47.78±1.10	45.16±0.89	42.55±1.20
A. porri	Pepper	51.74±2.82	51.49±2.87	50.52±2.62	49.63±2.42	48.34±2.17	48.32±1.81	45.87±1.27	45.33±1.21	44.48±1.08	43.09±0.82
A. japonica	Tomato	45.27±4.14	44.73±4.01	42.67±3.60	41.34±3.50	40.16±3.27	41.03±3.07	40.55±2.69	39.16±2.43	35.55±2.48	33.72±2.13

[a] Each value is a mean of three replicates. [b] Isolates were incubated at 28 °C ± 2 for six days.

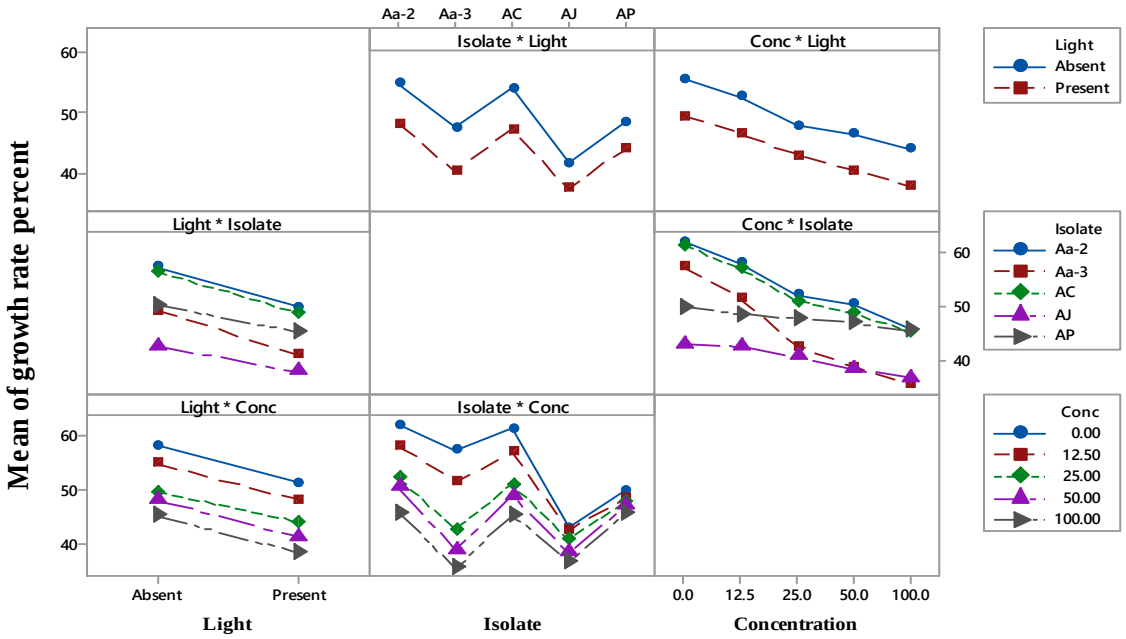

Figure 2. The linear growth under different isolates of *Alternaria* interactions between the main factors considered in this study.***Aa-2:** *A. alternata* isolated from tomato. **Aa-3:** *A. alternata* isolated from squash. **AC:** *A. compacta* isolated from pepper. **AJ:** *A. japonica* isolated from tomato. **AP:** *A. porri* isolated from pepper.

Table 3. Analysis of variance for linear growth using adjusted sum of squares for tests.

Source of variation		linear growth	
	df	F	*P*-value
Light [a]	1	108.33	0.000
Isolate [b]	4	60.78	0.000
TBO concentration [c]	4	54.39	0.000
Replicate [d]	2	0.01	0.990
Light*Isolate	4	1.25	0.291
Light*TBO concentration	4	0.16	0.956
Isolate*TBO concentration	16	3.87	0.000
Light*Isolate*TBO concentration	16	0.19	1.000
Error	398		
Total	449		

[a] Light source was either present or absent ,[b] *Alternaria compacata* isolated from pepper, *A. japonica* isolated from tomato, *A. porri* isolated from pepper, *A. alternata* isolated from tomato, and *A. alternata* isolated from squash. [c] Five concentrations were used: 0, 12.5, 25, 50, and 100 µg/µL of the TBO photosensitizer. [d] Three replicates were used for each treatment.

3.2. Production and assay of mycotoxin

In a preliminary test, the different isolates of *Alternaria* were investigated for the production of mycotoxin. *A. alternata* showed a considerable production of the mycotoxin alternariol, so the two isolates of this species were employed in this experiment. Alternariol was produced more and was highly significant in the *A. alternata* isolated from tomato with a mean of 26.45 compared to *A. alternata* isolated from squash with a mean of 14.46 µg/mL (Tables 4 and 5). The presence of light caused a significant decrease in the production of alternariol with a mean of 24.60 and 16.31 µg/mL in the case of absence and presence of light, respectively. Concerning toluidine blue O (TBO) concentration, the alternariol amount further decreased as the concentration increased, particularly in the presence of light. There was a significant difference among the concentrations (Table 5). Moreover, all the interactions among the different factors included in the analysis model were high and significantly different (Table 5).

Table 4. Effect of different concentrations of toluidine blue O (TBO) on the production of the mycotoxin, alternariol by *Alternaria allternata* isolated from squash or tomato.

	TBO concentration (µg/mL) (Mean±sd)									
Host	0		12.5		25		50		100	
	D *	L	D	L	D	L	D	L	D	L
A. alternata-squash	18.7±0.00	21.9±3.09	18.0±0.00	14.2±0.00	19.8±0.00	11.4±1.53	16.9±0.00	5.7±0.00	15.8±1.58	2.2±0.00
A. alternata-tomato	32.3±0.01	34.5±0.00	33.5±0.00	37.1±0.00	35.0±0.02	18.6±0.00	28.8±0.01	11.7±1.54	27.2±0.00	5.8±0.00

*D=TBO- treated inoculum of *A. alternata* was incubated in dark for one hour; L= TBO- treated inoculum of *A. alternata* was incubated in light for one hour.

Table 5. Analysis of variance for alternariol mycotoxin under different interactions.

Source	DF	Mean Square	F Value	Pr > F
Model	21	303.48	5.07E15	<.0001
Host [a]	1	2156.40	3.6E16	<.0001
Light [b]	1	1030.86	1.72E16	<.0001
Concentration [c]	4	450.69	7.53E15	<.0001
Replicate [d]	2	0.00	2.61	0.0865
Host*Presence or absence of light	1	35.11	5.87E14	<.0001
Host*concentration	4	62.43	1.04E15	<.0001
Light* Concentration	4	242.93	4.06E15	<.0001
Host*light*Concentration	4	31.61	5.28E14	<.0001
Error	38	0.001		
Corrected Total	59			

[a] Two hosts were studied: tomato and squash. [b] Treatments were divided into two groups: light-treated and dark-treated inoculums.

[c] The toluidine blue O (TBO) concentrations were used: 0, 12.5, 25, 50, and 100 µg/mL. [d] Three replicates were used for each treatment.

3.3. Production of Pectinolytic and Cellulolytic Enzymes

Pectinase and cellulase activities of both isolates of *Alternaria alternata* were significantly reduced when incubated in light as compared to dark (Table 6).

As the concentration of toluidine blue O (TBO) increased, the enzymes significantly decreased reaching 3.44 and 3.27 U/g substrate for pectinase and 1.66 and 1.47 U/g for cellulase at 100 µg/mL TBO incubated in the dark in the case of *A. alternata* isolated from tomato and squash, respectively (Table 6). The activity of both enzymes was steadily reduced when 100 µg/mL TBO-treated samples were exposed in light for sixty minutes reaching 1.03 and 0.73 U/g substrate for pectinase and 0.48 and 0.26 U/g substrate for cellulase in the case of *A. alternata* isolated from tomato and squash, respectively (Table 6). Generally, enzymes' activities were high in *A. alternata* isolated from tomato compared to *A. alternata* isolated from squash. Moreover, all the interactions among the different factors included in the regression analysis were high in significance (Table 7).

Table 6. Pectinase and cellulase activity (U/g substrate)* of *Alternaria alternata* isolates pre-incubated for 60 minutes with different concentrations of Toluidine Blue O (TBO) in dark (D) or in light (L).

alternata isolated from (host)	TBO concentration (µg/ml)									
	0		25		50		75		100	
	D	L	D	L	D	L	D	L	D	L
Pectinase										
Tomato	4.31±0.005	1.80±0.017	4.04±0.005	1.67±0.008	3.87±0.008	1.56±0.005	3.67±0.013	1.42±0.005	3.44±0.008	1.03±0.013
Squash	4.05±0.005	1.68±0.005	3.96±0.013	1.58±0.008	3.76±0.005	1.32±0.005	3.54±0.022	0.96±0.02	3.27±0.013	0.73±0.013
Cellulase										
Tomato	3.95±0.020	1.59±0.009	3.32±0.020	1.46±0.005	2.75±0.008	1.33±0.013	2.06±0.012	1.08±0.017	1.66±0.008	0.48±0.013
Squash	3.70±0.005	1.33±0.013	3.06±0.013	1.08±0.023	2.18±0.013	0.80±0.017	1.76±0.012	0.56±0.005	1.47±0.005	0.26±0.008

* One unit of enzyme activity is defined as the amount of enzyme required to release 1µmol reducing sugars per ml under assay condition.

Table 7. Analysis of Variance for enzyme activity, using adjusted sum of squares for tests.

Source	DF	Mean Square	F-Value	*P*-Value
Enzyme [a]	1	18.715	40197.62	0.000
Light [b]	1	120.683	259206.2	0.000
Isolate [c]	1	2.218	4764.36	0.000
Concentration [d]	4	6.017	12924.16	0.000
Replicate [e]	2	0.001	1.38	0.257
Enzyme*Light	1	5.059	10865.37	0.000
Enzyme*Isolate	1	0.165	354.37	0.000
Enzyme*Concentration	4	0.684	1468.54	0.000
Light*Isolate	1	0.053	113.66	0.000
Light*Concentration	4	0.421	905.12	0.000
Isolate*Concentration	4	0.036	78.15	0.000
Enzyme*Light*Isolate	1	0.002	4.04	0.048
Enzyme*Light*Concentration	4	0.449	964.19	0.000
Enzyme*Isolate*Concentration	4	0.035	75.65	0.000
Light*Isolate*Concentration	4	0.023	48.96	0.000
Enzyme*Light*Isolate*Concentration	4	0.007	15.48	0.000
Error	78	0.000		
Total	119	177.624		

[a] Two enzymes were studied: cellulase and pectinase. [b] Light source was either present or absent. [c] Tow isolates of *Alternaria* were used: *A. alternata* isolated from tomato, and *A. alternata* isolated from squash. [d] Five concentrations were used: 0, 12.5, 25, 50, and 100 µg/µl of the TBO photosensitizer. [e] Three replicates were used for each treatment.

4. Discussion

Many dematiaceous hyphomycetes cause economically critical diseases in all types of vascular plants, especially agricultural crops. The class hyphomycetes are primary pathogens of plants and weeds, causing, root, stem and leaf necrosis, dieback, cankers, wilts, and blight (Ellis and Ellis, 1987).

In the present study, thirty fungal isolates were recovered from tomato, squash and pepper samples including fruits, leaves and stems cultivated in different regions at Almadinah Almunawwarah. The identification was based on the morphological characteristics of the isolates, and was confirmed by DNA sequencing and BLAST analysis. The isolates were related to six species assigned to five genera. *Alternaria japonica*, *A. porri*, and *A. compacta*, are new records for Saudi Arabia. About 50 % of the isolated species were related to *A. alternata*. This specie is a quite common pathogenic fungus associated with vegetables. *A. alternata* is considered as an opportunistic pathogen on numerous hosts causing leaf spots, rots, and blights on many plant parts (Silva and Melo, 1999). Other isolated members of *Alternaria*, though recovered in low occurrence, included *A. compacta*, *A. porri* and *A. japonica*. Several authors indicated that the release of many dry-dispersed spores, including *A. porri*, shows diurnal periodicity, with the majority of spores being collected during midday when temperature, wind speed and the level of turbulence near the ground are highest, and relative humidity lowest (Humpherson-Jones and Phelps, 1989). This occurrence conditions of *Alternaria* spp. match the environmental conditions dominant at Almadinah region.

A. japonica and *A. compacta* are rarely encountered as phytopathogens. The former species were identified from

the leaf lesions of turnip and cabbage plants in Spain (Bassimba *et al*., 2013), and from the seeds of cruciferous vegetable crops in Japan (Tohyama and Tsuda, 1995), and from the Chinese cabbage seedlings in China (Ren and Zhang, 2012), while the later fungal species were recovered from climbing hydrangea (*Hydrangea anomala* subsp. petiolaris) (Garibaldi *et al.*, 2008). *Phoma tropica* has been identified as thermotolerant species, which is mainly found in greenhouses on a wide range of hosts, but probably has a tropical origin (Schneider and Boerema, 1975). *Phoma* species are common soil inhabitants. Overhead irrigation or rain splash may result in an excessive spread of the species.

Thielavia arenaria, isolated in the present research, may be interrelated to some other species especially those producing dark pigmented mycelium and brownish-black, hairy ascomata like *T. gigaspora* Mouchacca, *T. hyrcaniae Nicot*, *T. microspora* Mouchacca and *T. subthermophila* Mouchacca. (Mouchacca, 1973). The genotype of *T. subthermophila* is very close to *T. arenaria*, revealed in dendogram of the fungal isolates recovered in this study, and amplified using the ITS4/5 gene region (Figure 5). Thermotolerance tests of *T. arenaria* (unpublished data) showed that the isolate grew rapidly at both 35°C and at higher temperatures of 42°C, 45°C, and 50°C. This ecological manner is harmonizing with the environmental conditions predominant in Almadinah region during summer.

Each of *Podospora* and *Chaetomium*, was isolated once in the current study. These two genera are considered as soil mycobiota of date palm plantations in Elche, SE Spain (Abdullah *et al.*, 2010), a situation similar to that found in Saudi Arabia where vast areas are abundant with date palm trees. The investigations on soil fungi of several date palm plantations in Iraq have revealed several novel and interesting fungal taxa (Abdullah and Zora, 1993).

The present work is focused on the possibility of inactivating the phytopathogenic fungi by exploiting photosensitization. Toluidine Blue O (TBO) in different concentrations up to 100µg/mL was employed as photosensitizer. The application of higher TBO concentration (>100µg/mL) was significantly ineffective in exerting more reduction in growth when compared with that recorded at 100µg/mL (unpublished data). The decrease in the efficacy of the photosensitizer at the higher concentration can be explained by the decreased penetration of light through the suspension with increasing the TBO concentration rather than the decrease in the uptake. This would reduce the amount of light reaching the sensitized fungi, and would reduce the overall efficiency of the system (Manpreet *et al*., 1998).

Five of the isolated fungi were the target of the experiments, namely: *Alternaria alternata* (two molecularly different isolates, one isolated from tomato and one from squash), *A. compacta*, *A. porri* and *A. japonica*. The linear growth and spore germination of the investigated isolates were gradually decreased as the TBO concentration elevated up to100µg/mL. The reduction in growth and in spore germination was more obvious during the pretreatment of the samples with TBO in the presence of light for sixty minutes. *A. japonica* and *A. alternata* isolated from squash were more susceptible than the other isolates. Minnock *et al.* (1996) reported that gram-negative and gram positive bacteria can be killed after sensitization with phthalocyanine using 400 w metal halide spectral lambs. Ladan *et al.* (1993) demonstrated that Staphylococcus aureus can be killed by using a white light source and hematoporphyrin as a photosensitizer.

A. alternata is a common species of critical significance because it produces a number of mycotoxins. Natural occurrence of alternariol, alternariol methyl ester and other toxins has been frequently detected in sunflower seeds, apples, mandarins, olives, spices, tomatoes, soybean, cereals, melons, peppers, apples, pecans, wheat flour, oilseed rape meal, cucumbers, oranges, lemons as well as processed fruits and vegetables (Scott and Kanhere, 2001 and Asam *et al.*, 2011).

A steady reduction in the alternariol production for both isolates was noticed in case of the treatment of the inoculums with TBO; they were exposed to light for 60 minutes reaching 2.2 and 5.8 µg/mL for *A. alternata* isolated from squash and tomato, respectively as compared to 21.9 and 34.5 µg/mL regarding control in the absence of light. The results indicate that the reduction in alternariol may be attributed to the presence of the photosenistizer. This result is somehow consistent with that published by Söderhäll *et al*. (1978) who found that the production of alternariol and alternariol monomethyl ether by *A. alternata* (Fr.) Keissler, grown in drop culture, produced alternariol and alternariol monomethyl ether were almost completely inhibited when the fungal cultures were exposed to white light (180 W/m2), although mycelial dry weight was not significantly affected. However, the divergence in the results of Söderhäll *et al.* (1978) and those cited in the present work might be related to the fungus long exposure to light; its photosensitization may be attributed to the presence of melanin pigment. Häggblom and Unestam (1979) showed that light inhibits the production of the mycotoxins alternariol and alternariol monomethyl ether, both polyketids produced by *A. alternata*. The authors also indicated that the effect seems to be general because seven of the isolates of *A. alternata* with different alternariol- and alternariol monomethyl ether-producing abilities, all respond to continuous light with reduced levels of alternariol and alternariol monomethyl ether. DiCosmo and Straus (1985) indicated that the mycotoxin alternariol is phototoxic to *Escherichia coli* in the presence of near UV light (320-400 nm). The phototoxicity bioassays with a DNA repair-deficient mutant of *E. coli* suggested that DNA may be the molecular target for photo-induced toxicity of alternariol. Interactions between alternariol and double-stranded, supercoiled DNA suggest that alternariol interacts with DNA by intercalation. These results suggest that alternariol is a phototoxic, DNA-intercalating agent and is a DNA cross-linking mycotoxin in near UV light.

Phytopathogenic fungi that attempt to colonize higher plants must compete with physical barriers of the host as surface waxes and cell wall (Dyakov *et al.*, 2007). Phytopathogenic fungi produce several pectolytic enzymes, which are capable to degrade the plant cell wall components during plant pathogenesis. It was proved that hydrolytic enzymes have a significant role in pathogenesis due to their impact on the degradation of infected tissues and the expansion of disease (Bateman and Miller, 1966). However, some investigators have not proved that these enzymes are specific factors determining the degree of phytopathogenic virulence (Keen and Erwin, 1971). On

the other hand, some authors indicated a correlation between virulence of isolates and their ability to produce pectolytic enzymes (El-Ktatny, 1984). In a number of systems, a strong correlation has been found between the presence of pectinolytic enzymes and the disease symptoms and disease virulence (Durrands and Cooper, 1988).

On the whole, the mean activity of pectinase exhibited higher activity than cellulase for both *A. alternata* isolates. It is well-known that the relative quantitative production of the enzyme depends on the availability of the substrate in the cell wall of the host plant that provides all the necessary nutrients for the pathogen. The xylanase, pectinase and cellulase yields by *Sporotrichum thermophile* were 400-, 200-, and 20-fold higher in solid state fermentation as compared to those in submerged fermentation and similar increase in yields of enzymes in solid-state fermentation over submerged fermentation (Babu and Satyanarayana, 1995).

Steady inhibition of both investigated enzymes was recorded for the mycelium developed from photosensitized inoculums in the presence of light at 100 µg/ml TBO concentration. The activity of pectinase reached 1.01 and 0.72 while dropped down to 0.43 and 0.29 U/g substrate, in the case of *A. aternata* isolates recovered from tomato and squash, respectively. Paardekooper *et al*. (1995) showed that the photodynamic treatment of yeast with TBO causes a rapid uptake of a small amount of the dye, resulting in damage to intracellular enzymes (alcohol dehydrogenase, cytochrome-c oxidase, glyceraldehydes-3-phosphate dehydrogenase and hexokinase). These enzymes control the glycolysis and oxidative phosphorylation. It was found that when the *Kluyveromyces marxianus* is pretreated with 1 µM of chloroaluminium phthalocyanine and then illuminated with 150 W tungsten-halogen slide projector lamp, this did not affect the hexokinase activity, but the activity of the mitochondrial enzyme decreases. Ouf *et al.* (2003) evaluated the antifungal activity of solar simulator in presence of haematoporphyrin derivative, methylene blue and toluidine blue O as photosensitizers against seven dermatophytes. They indicated drastic inhibition of keratinase, phosphatases, amylase and lipase when the fungi were irradiated after their treatment with the photosensitizers. *Trichophyton verrucosum* and *T. mentagrophytes* were the most sensitive to photo-sensitization.

The relatively lower efficiency of TBO in light against *A. alternata* at 100 µg/mL compared to the corresponding lower concentrations or to that exposed to TBO in light may be due to the high density of the TBO particles which decrease light penetration of the photosensitizer. Moreover, the high concentration of TBO may increase the particles aggregation and consequently lowers fungitoxic effect. This phenomenon is known as self-shielding, in which saturated concentrations of the substance absorb a major portion of the incident light in the surrounding medium and superficial layers. A study by Rizvi *et al* (2013) stated that high concentrations of the photosensitizer may not induce the expected effective result. These observations point to the importance of "right" amount of photosensitizer and "right" light irradiance to achieve effective treatment.

5. Conclusions

It was possible using photosensitizers, such as the toluidine blue O (TBO), for the protection of plants against phytopathogenic fungi, above all, *Alternaria* spp. The photosensitizer (TBO) is useful as an alternative method in plant disease management and disease control in Saudi Arabia, a country characterized by sunshine throughout the year. When compared to the classical and traditional methods such as the usage of fungicides which cause environmental pollution and more significantly serious health hazards to human beings, the photosensitizers, such as (TBO), are safer, more economical, and eco-friendly.

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The Efficacy of the Plant Extracts of *Afrostyrax kamerunensis*, *Monodora myristica*, *Moringa oleifera* and *Azadirachta indica* against the Infestation of the Leather Beetle, *Dermestes maculatus* De Geer in Smoked African Mud Catfish, *Clarias gariepinus* Burchell

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Abstract

The information that the leather beetle, *Dermestes maculatus* De Geer is globally less susceptible to chemical insecticides provided the rationale to test some cosmopolitan elite plant materials on the pest. Extracts from four plant materials (country onion, *Afrostyrax kamerunensis* Perkins and Gilg; African nut meg, *Monodora myristica* Dunal; moringa, *Moringa oleifera* Lam; and neem, *Azadirachta indica* A. Juss) at 2.5 and 5.0 mL/ 100 g fish were assessed under tropical storage conditions (temperature: 31.9^{o}C; relative humidity: 68.3 %) to control the leather beetle, *Dermestes maculatus* infesting smoked African mud catfish, *Clarias gariepinus* Burchell. Four important indices in insect pest control were assessed using standard storage entomology procedures. Each of the plant extracts (excluding *M. oleifera*) at 5.0 ml/ 100 g fish caused significantly high mortality in *D. maculatus* adults at third and fourth day exposure periods. Adult beetle emergence was absolutely inhibited in the catfish treated with *A. kamerunensis*, *M. myristica* and *A. indica* at both test concentrations. Weight loss due to insect infestation was suppressed significantly, also when compared with untreated control. The test botanicals were effective in this order, *A. indica* > *M. myristica* > *A. kamerunensis* > *M. oleifera*. *Dermestes maculatus* adults were most sensitive to the repellent action of *A. kamerunensis*. Therefore, the study has identified a plant material that repels *D. maculatus* adults better than neem. These findings revealed that the extracts of *A. indica*, *M. myristica* and *A. kamerunensis* could be incorporated into post-harvest fish management strategies against *D. maculates*, particularly in solving the problem of the development of resistance to chemical insecticides.

Keywords: *Afrostyrax kamerunensis*, Botanicals, *Dermestes maculatus*, Fish management, Repellent, Resistance.

1. Introduction

Fish is highly nutritious and emphasis on the health benefits of fish consumption is on the increase (Nwosu *et al*., 2016). Fish is an important component of several delicacies in Nigeria and other countries of the world. Nowadays, catfish is highly appreciated in Nigeria. Rearing catfish has both aesthetic and economic benefits. It serves as a valuable source of income through self-reliance and employment. Catfish trading alleviates poverty, yields foreign exchange earnings, and provides raw material for the feed industry (Akinwumi *et al*., 2007). Generally, it has been estimated that the annual fish consumption in Nigeria is 1.2 million metric tons (FDF, 2005). The possibility of having fish supply that meets this demand is undermined by insect pest infestations. After harvest, fish is often processed, and sometimes stored for

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inevitable reasons of requiring a longer time to make sales, and to have a stock for consumption for a relatively longer period. Being highly perishable, catfish (like any other fish) must be treated to be relatively durable. One of such important treatments given to fish especially in developing countries is smoking (Nwosu *et al*., 2016). It has been indicated that 45 % of the total fish catch in Nigeria is utilized as smoked fish (FAO, 2002). Unfortunately, even smoked fish is susceptible to both quantitative and qualitative losses. *Dermestes maculatus* infestations contribute substantially (71.5 %) to the heavy losses in smoked catfish (Osuji, 1974; Akinwumi *et al*., 2007) and this calls for quick action.

Chemical insecticides can be used against *D. maculatus*; however, the risks involved outweigh the benefits. Health hazards, eco-toxicity, loss of visual appeal due to impacting influence of chemicals and exorbitant cost prices of these synthetic chemical insecticides are the major problems associated with their general use to suppress infestations and damage by *D. maculatus* in stored catfish (Boeke *et al*., 2001; Akinwumi *et al*., 2007). Notably, it has been reported that unlike other insect pests of stored products, dermestid larvae and adults are less susceptible to chemical insecticides (Amusan and Okorie, 2002; Onu and Baba, 2003). This observation is the major justification for the present testing of widely-available botanicals with a view to identify those that can tackle the problem of resistance to chemical insecticides and thus, effectively protect stored catfish against *D. maculatus* attack. In a previous study by Keeler (1999), neem (*Azadirachta indica*) products (botanicals) were tested for toxic, growth regulatory and antifeedant effects against *D. maculatus*, under similar conditions in storage facility and the result is part of the motivation in this study for testing the four plant materials against *D. maculatus*.

Indeed, for more than a century, plant-derived insecticides such as derris, sabadilla, nicotine, pyrethrum, physostigmine and rotenone have been in use (Adedire and Lajide, 1999). Similarly, a host of plant species in Nigeria have been used as control agents for various pests (Lale, 1995). Information in literature favors the use of plant-derived insecticides to control especially, *D. maculatus* (Amusan and Okorie, 2002; Onu and Baba, 2003). In the present study, parts of some cosmopolitan plant species (readily available) were tested against *D. maculatus*, notorious for devastating stored dried fish (Chris *et al*., 2014).

2. Materials and Methods

2.1. Insect Culture

Population of *D. maculatus* was first obtained from naturally-infested smoked catfish. The leather beetles were cultured in a Kilner jar covered with white muslin cloth and were routinely maintained at laboratory average temperature (30.9 ^{o}C) and relative humidity (68.3 %). To have new progenies for the experiment, adult beetles from the stock colony were placed on fresh disinfested fish for feeding and oviposition. Water-soaked cotton wools were put in the jar to induce oviposition and parent adults were removed after twenty-one days (Akinwumi *et al*., 2007).

2.2. Plant Materials and Extracts

The plant materials used in this study were the bulb of country onion, *Afrostyrax kamerunensis* Perkins and Gilg (Huaceae); seeds of the African nut meg, *Monodora myristica* Dunal (Annonaceae); seeds of moringa, *Moringa oleifera* Lam (Moringaceae) and seeds of neem, *Azadirachta indica* A. Juss (Meliaceae). All the plant materials are available in Nigeria; they have medicinal value, and are unlikely to have adverse effects on human health. The method reported by Akinwumi *et al*. (2007) was employed in the preparation of plant materials and extracts. The botanicals were dried in an electric oven at 40 ^{o}C for a period of eight hours. Thereafter, they were ground thoroughly using an electric blender (5.0 HP), and were made to pass through a-40 holes mm^{-2} mesh screen. Ten g of each of the sieved plant materials were put into a round bottom flask, and 100 ml of absolute ethanol was added and soaked for twenty-four hours. The mixture was boiled at 60 ^{o}C for thirty minutes in the laboratory water bath. The solution was filtered using Whatman no.1 filter paper. The filtrates were kept in separate and tightly-covered bottles prior to use.

2.3. Efficacy Test of Extracts

The samples of smoked African mud catfish, *C. gariepinus* (weighing average of 100 g) used for the assay were obtained from Northbank Market, Makurdi, Benue State, Nigeria. The fish samples and the experimental jars were disinfested by heat treatment in the Gallenkamp oven at 60 ^{o}C for one hour, and were allowed to cool at room temperature prior to commencement of the assay (Akinwumi *et al*., 2007). An aliquot of 2.5 mL of each of the four plant extracts was evenly rubbed separately to four disinfested smoked catfish samples. The treated fish samples were air-dried for two hours in order to eliminate traces of the solvent, and were then placed in four separate plastic jars (depth: 8 cm; diameter: 10 cm). An untreated control was designated. Ten newly emerged adults of *D. maculatus* were introduced into the five different jars, and were covered with muslin nets for ventilation and protection. A similar set-up using 5.0 mL of each of the plant extracts was made. The experimental design was randomized complete block design with four replications by members of the research group in different regions of Nigeria, namely North (Benue State), West (Osun State), South-East (Imo Sate) and South-South (Rivers State). The number of dead *D. maculatus* adults was recorded daily for four days, and the mortality rate was calculated. Beetles were confirmed dead when they failed to respond to probing with a sharp pin at the abdomen. All dead and live beetles were sieved out immediately after mortality count to ensure that the emerging adults were a direct consequence of the number of eggs oviposited in four days (Ileke *et al*., 2012). After mortality check, the set-up was maintained under the same experimental storage conditions until thirty days later when the accumulated emergence of *D. maculatus* in the treated and untreated smoked catfish samples was assessed. On the 30th day (following mortality count), the fish samples were reweighed using a digital balance to evaluate the weight loss resulting from *D. maculatus* infestation.

2.4. Repellence Test

A chamber of 25 x12 x 10 cm was constructed for the investigation on repellence (Akinwumi *et al*., 2007). An aliquot of 5.0 mL of each of the plant extracts was thoroughly rubbed separately on the skin of four randomly-selected fish samples weighing about 100 g. An untreated fish (of same weight) was included in the experiment. The treated and untreated samples were placed separately at each edge of the chamber, 10 cm apart. This was immediately followed by the introduction of ten *D. maculatus* adults (2 – 4 days old) at the center. The adult insects used in this segment of the study were also starved for forty-eight hours. Daily observations were made for five days immediately after application and the number of *D. maculatus* adults found on or within a 1.0 cm radius of treated and untreated fish was recorded twice daily at 09:00 hours and 16:00 hours. The average count for each five-day period was expressed as a percentage of repellency and the results (for treated fish only) were assigned to a repellency class using the following scale described by Laudani *et al*. (1955). Class 0, < 0.1 %; class I, 0.1 – 20 %; class II, 20.1 – 40 %; class III, 40.1 – 60 %, class IV, 60.1 – 80 %; class V, 80.1 – 100%. Repellency was re-assessed one month later.

2.5. Statistical Analysis

Data were investigated for normality using both residual and box plots. The assumption for homogeneity of group variance was tested using Levene's test for equality of variances. If data were normally distributed, and the assumption of homogeneity of group variance was met, or the deviation from homogeneity assumption was not sufficiently strong to affect the results (Sulehrie *et al*., 2003), the data were subjected to one-way analysis of variance. When the F-test was significant, treatment means were separated using a more pragmatic multiple comparison test, Honestly Studentized range (HSD). Inference was made at $\alpha = 0.05$. The statistical software was SPSS (Statistical Package for the Social Sciences) (version 19.0).

3. Results

Results of *D. maculatus* mortality due to the treatment with four plant extracts are summarized in Table 1. All the plant species were toxic to the leather beetle. However, the number of *D. maculatus* adults killed by the different plant extracts was statistically different. The extract of *A. indica* was the most toxic to *D. maculatus* after ninety-six hours of exposure. At a dose of 2.5 mL/100 g fish, *A. kamerunensis* and *M. myristica* showed faster action within the first forty-eight hours. At a higher dose of 5.0 ml/100 g fish, *A. indica* recorded the fastest action; killing 74 % of the dermestids in seventy-two hours. The extract of *M. oleifera* was the least toxic to the leather beetles, even at an increased dose of 5.0 mL/100 g fish. In general, mortality increased progressively with increasing application rate and time of exposure. At both test concentrations, it was observed that the extracts of the plant materials (except *M. oleifera*) totally inhibited the emergence of *D. maculatus* adults from smoked catfish within thirty days of storage (Table 2). Inability to prevent adult emergence was significant for *M. oleifera*.

Table 3 presents the effect of the plant extracts on the weight loss of the treated fish. Decrease in weight loss was not consistently recorded when fish was treated with a higher concentration of 5.0 mL. Each of the plant materials allowed a certain level of weight loss among the treated specimens; inclusive of extracts that completely inhibited adult emergence. Extracts of *A. indica* and *M. myristica* were best at disallowing loss in the quantity of stored smoked catfish infested by leather beetles. Table 4 shows the spectrum of repellence against adult *D. maculatus* offered by extracts of different plants. *Afrostyrax kamerunensis* is the best repellant, followed by the seed extracts of *M. myristica* and *A. indica*, which came second in repelling adult leather beetles from infesting smoked catfish. Meanwhile, *D. maculatus* adults were the least sensitive to the repellent action of the seed extract of *M. oleifera* under the tropical storage conditions investigated.

Table 1. Effects of four plant extracts on the mortality of adult *D. maculatus* De Geer infesting smoked cat fish, *C. gariepinus* Burchell (in all cases, df = 4,15).

Plant materials	% mortality at 2.5 mL/100 g fish				% mortality at 5.0 mL/100 g fish			
	24 h	48 h	72 h	96 h	24 h	48 h	72 h	96 h
A.kamerunensis	13.33±4.70^{a}	33.33±0.05^{a}	36.67±0.43^{a}	60.00±0.00^{a}	16.67±0.50^{a}	33.34±0.01^{a}	56.67±0.41^{a}	86.67±2.67^{a}
M. myristica	16.67±0.66^{a}	40.00±0.00^{a}	46.67±0.02^{a}	66.67±0.11^{a}	30±0.00^{b}	43.33±0.29^{a}	73.33±0.67^{b}	96.66±0.45^{a}
M. oleifera	6.67±0.10^{b}	10.00±0.00^{b}	16.67±0.55^{b}	30.00±0.00^{b}	10.00±0.00^{a}	13.33±3.90^{b}	36.66±0.01^{c}	49.99±0.01^{b}
A. indica	3.33±0.10^{b}	20.00±0.01^{c}	46.66±0.30^{a}	86.66±0.01^{c}	16.67±0.33^{a}	43.34±0.89^{a}	74.34±2.50^{b}	97.67±0.02^{a}
Control	0.00±0.00^{b}	0.00±0.00^{d}	0.00±0.00^{c}	0.00±0.00^{d}	0.00±0.00^{c}	0.00±0.00^{c}	0.00±0.00^{d}	0.00±0.00^{c}
F	5.3	22.1	10.7	19.0	14.5	12.9	32.2	18.9
P	0.03	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01	< 0.01

Data are means ± standard error of the means of four replications

Means in a column followed by the same letter are not significantly different by HSD ($\alpha = 0.05$)

Table 2. Effects of four plant extracts on the adult emergence of *D. maculatus* De Geer infesting smoked cat fish, *C. gariepinus* Burchell (in all cases, df = 4,15) within 30 days.

Plant materials	Number of emerged adults (at 2.5 mL/100 g fish)	Number of emerged adults (at 5.0 mL/100 g fish)
A. kamerunensis	0.00±0.00[a]	0.00±0.00[a]
M. myristica	0.00±0.00[a]	0.00±0.00[a]
M. oleifera	37.53±7.10[b]	41.96±3.20[b]
A. indica	0.00±0.00[a]	0.00±0.00[a]
Control	93.18±10.20[c]	93.18±10.20[c]
F	45.2	38.3
P	< 0.01	< 0.01

Data are means ± standard error of the means of four replications

Means in a column followed by the same letter are not significantly different by HSD (α = 0.05)

Table 3. Effects of four plant extracts on the weight loss caused by *D. maculatus* De Geer infesting smoked cat fish, *C. gariepinus* Burchell (in all cases, df = 4,15).

Plant materials	% weight loss (at 2.5 mL/100 g fish)	% weight loss (at 5.0 mL/100 g fish)
A. kamerunensis	0.63±0.37[a]	0.30±0.00[a]
M. myristica	0.07±0.50[b]	0.25±0.01[a]
M. oleifera	3.07±0.10[c]	3.90±1.20[b]
A. indica	0.04±0.00[b]	0.27±0.00[a]
Control	4.01±0.20[c]	4.01±0.20[b]
F	8.6	5.5
P	< 0.02	< 0.02

Data are means ± standard error of the means of four replication

Means in a column followed by the same letter are not significantly different by HSD (α = 0.05)

Table 4 Repellent action of four plant extracts (5.0 mL/100 g fish) on adults of *D. maculatus* De Geer infesting stored catfish, *C. gariepinus* Burchell

Plant materials	1 - 5 days after application		1 month after application	
	% Repellence	Repellence class	% Repellence	Repellence class
A. kamerunensis	50.45±2.90	III	50.01±0.01	III
M. myristica	39.67±0.12	II	30.52±0.34	II
M. oleifera	8.77±5.34	I	9.02±0.10	I
A. indica	37.64±0.01	II	36.33±2.61	II

Class 0, < 0.1 %; Class I, 0.1 – 20 %; Class II, 20.1 – 40 %; Class III, 40.1 – 60 %, Class IV, 60.1 – 80 %; Class V, 80.1 – 100%.

4. Discussion

The results of this investigation revealed that the extracts of neem, *Azadirachta indica* is the best bio-insecticide that controlled *D. maculatus* adults because it killed the highest number of adult leather beetles at the end of the exposure period. The analyses of results further showed that the extracts of *A. indica*, *M. myristica* and *A. kamerunensis* inhibited adult emergence totally, while the extracts of *A. indica* and *M. myristica* allowed the lowest quantitative fish loss. It was also revealed that the extracts of *A. indica* and *M. myristica* were next to *A. kamerunensis* in repelling the insects. The best performance of neem in terms of causing mortality of *D. maculatus* adults is in agreement with the findings of Keeler (1999). According to the literature, at various test concentrations of neem products, the botanical caused high mortality of *D. maculatus*, had antifeedant effect on the insect larvae which consequently failed to develop to the pupal stage, and the adult emergence was totally inhibited. According to the information in the literature, the repellence property of neem is not as strong as the toxic and antifeedant properties. However, repellence has been reported as a major mechanism by which plant materials evoke control on stored product insect pests (Akunwumi *et al*., 2007). This is supported by the findings of this study. The active ingredient in *A. indica* is largely *azadirachtin* (Xie *et al*., 1995). In other words, azadirachtin was largely responsible for the toxic (physiological), inhibitory (physiological) and repellent (behavioural) actions of neem on *D. maculatus*. From the analyses of results, the seed extract of *M. myristica* was also effective in managing *D. maculatus* infestations of smoked catfish. The biological activity of *M. myristica* is strongly attributed to terpenes and linoleic acids (Akinwumi *et al*., 2007). On the whole, plant extracts are highly lipophilic (Lale, 1995) and thus, they have the capacity to penetrate the insect integument. Previous studies reported the effectiveness of *M. myristica* and *A. indica* in the control of some stored product insect pests (Fasakin, 2003, Akinwumi *et al*., 2007). The poor insecticidal activity of the extract of *M. oleifera* tallied with the information in the current literature. Irikannu *et al*. (2015) observed that the seed oil extract of *M. oleifera* had no obvious insecticidal effect on some stored product insect pests which was also observed in this study.

5. Conclusion

In conclusion, although the medicinal value of *M. oleifera* has been reported (Lakshmipriya *et al*., 2016), in this study, it failed to control *D. maculatus* infesting smoked catfish. At the application rates examined, it can be concluded that the seed extract of *M. oleifera* is not recommended for use in the protection of smoked catfish against *D. maculatus* infestation under tropical storage conditions. This study has included *D. maculatus* in the range of stored-product insect pests controllable by the extracts of *A. indica* and *M. myristica* seeds. Thus, they can substantially be of help in the bio-rational management of *D. maculatus* in stored smoked catfish. It is encouraging to note that the two effective botanicals possess medicinal values and do not have adverse effects on human health. Therefore, it can be inferred that the topical applications of these plant materials will remedy the problem of resistance associated with the use of chemical insecticides in *D.*

maculatus control, and prevent the health and environmental risks also associated with chemical insecticides. The effects of *A. kamerunensis* and *M. myristica* were more noticeable than the effect of *A. indica* within the first forty-eight hours; however, they were soon overtaken by *A. indica* which ultimately killed more dermestids. In terms of repellence, *A. kamerunensis* (with a cumulative lower mortality effect) ranked first and that is an edge over neem.

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Mitigation of Alpha-Cypermethrin-Induced Hepatotoxicity in Rats by *Tribulus terrestris* Rich in Antioxidant Compounds

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Abstract

Tribulus terrestris is used traditionally as a medicinal herb to improve the sexual functions. However, its hepatoprotective effects against alpha-cypermethrin (an insecticide) hepatotoxic effects are still not fully elucidated. The present study investigates for the first time the hepatoprotective effect of the *T. terrestris* extract against alpha-cypermethrin induced liver toxicity in addition to its phytochemical composition and *in vitro* antioxidant activity. Several phenolic and flavonoids' compounds (ellagic and ferulic acid, hesperidin and quercetrin) were screened in the *T. terrestris* extract by HPLC. The *T. terrestris* extract exhibited *in vitro* antioxidant activity using $DPPH^{\bullet}$, $ABTS^{\bullet+}$, and reducing power assays. The remarkable effects of the *T. terrestris* extract in the attenuation of hepatotoxicity induced by α-cypermethrin in rats are investigated for the first time in the current study. The administration of the *T. terrestris* extract decreased liver enzymes; alanine amino transferase (ALT), and aspartate amino transferase (AST). Also, it increased antioxidant; glutathione (GSH) and paraoxnase-1(POX-1) enzyme, and decreased oxidant; malondialdehyde (MDA) and nitric oxide (NO). Furthermore, it decreased liver inflammatory markers; tumor necrosis factor alpha (TNF - α), adiponectin and lipocalin. The hepatoprotective effect of *T. terrestris* extract could be attributed to its ability to hunt free radicals and induce the antioxidant enzymes expression in addition to the down-regulation of pro-inflammatory markers in liver injuries.

Keywords: α-Cypermethrin, Hepatotoxicity, HPLC, Polyphenolic compounds, *Tribulus terrestris*.

1. Introduction

Pesticides are toxic chemicals, which contaminate the entire environment including air, soil, water, and have been detected in human and animal tissue samples all over the world (Carvalho, 2017). Prolonged exposure to toxic pesticides has shown harmful effect to the skin, eyes, liver, the gastrointestinal tract, kidneys, the reproductive system, nervous system, cardiovascular system, blood and the endocrine system (IPCS, 2010).

Liver is the main target organ for drugs, xenobiotics, and other toxic chemicals because of the liver's complex anatomical texture, different metabolic functions, and direct association with the gastrointestinal tract. Pesticides including cypermethrin are aggressive chemicals that cause liver damage including hepatic fibrosis, cirrhosis, steatosis and inflammation (Cataudella *et al.,* 2012).

Alpha Cypermethrin (α-CYP), a synthetic type II pyrethroid insecticide, is used extensively in different countries for pest control, because of its high influence against a wide range of insects and because of its low toxicity to mammals (Abdou *et al.,* 2012). Despite the low toxicity of α-CYP, its accumulation in various food chains and persistence in different mammalian tissues including the liver, kidney, and the brain are the reasons behind its higher toxic levels (Yavasoglu *et al.,* 2006; Singh *et al.,* 2012). It induces mitochondrial dysfunction and oxidative stress especially lipid peroxidation in animal models, resulting in both an elevation of oxidative stress markers and a reduction in antioxidant activities (El-Demerdash, 2011). Moreover, Yavasoglu *et al.* (2006) reported that α-CYP treatment increased the apoptotic index in the liver of rats, and it might cause hazardous effects in different levels to non-target organisms.

Several studies have reported that the pretreatment with different plant extracts as a source for natural antioxidants can alleviate the side effects and toxicity of cypermethrin in rats (Sushma and Devasena, 2010; Abdou *et al.,* 2012).

Tribulus terrestris L. is an annual flowering creeping plant that belongs to the family Zygophylaceae, which is native to the Mediterranean region. However, it grows widely in the warm regions of Africa, Asia, America, Europe, and Australia (Qureshi *et al.,* 2014). It is known by different Arabic names: Ders El-Agouz, Hasak, Qutiba and Al-Gutub (Al-Ali *et al.,* 2003), with some common names such as caltrop, goat head, puncture vine, bull's head, devil's thorn, and ground burr nut (Kostova and

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Dinchev, 2005). In Egypt, China, India, and other countries, it is used traditionally as a medicinal herb to improve sexual functions in both sexes (Kamenov *et al.*, 2017). Also, anticancer (Kim *et al.*, 2011), antibacterial (Soleimanpour *et al.*, 2015), antifungal (Zhang *et al.*, 2005), hypoglycemic (El-Shaibany *et al.*, 2015) effects and antioxidant activities (Hammoda *et al.*, 2013) of different extracts of *T. terrestris* have been reported. The phytochemical investigation of *T. terrestris* revealed that it contains different active compounds including saponins (Kostova and Dinchev, 2005; Hammoda *et al.*, 2013), alkaloids (Wu *et al.*, 1999), flavonoids and sterols (Ivanova *et al.*, 2011).

To the authors' knowledge, there are no reports on the hepatoprotective effect of *T. terrestris* especially against α-cypermethrin, an insecticide used widely in Egypt. Because of the previously reported hepatic free radical-mediated tissue damage and dysfunction effect of alpha-cypermethrin in rats, these findings intrigued authors to investigate the phytochemical composition of *T. terrestris* extract. In addition, its hepatoprotective effect against alpha-cypermethrin-induced liver injury in rats in the context of its antioxidant and anti-inflammatory properties were also assessed.

2. Materials and Methods

2.1. Materials

2.1.1. Chemicals

Alpha-cypermethrin (10 % EC) was purchased from KZ pesticides company, Egypt. $ABTS^{\cdot+}$ (2, 2'-azinobis (3-ethyl benzothiazoline - 6 - sulfonic acid)), Folin-Ciocalteau reagents, Gallic acid, quercetin, $DPPH^{\bullet}$ (2, 2-diphenyl-1-picrylhydrazyl), BHT: Butyl Hydroxy toluene and, potassium ferricyanide, Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) were directly purchased from (Sigma Chemical Co., St. Louis, MO, USA).

2.1.2. Plant Samples

Tribulus terrestris aerial parts were collected from National Research Centre farm in Giza, Egypt. They were air-dried and then milled by a mixer grinder to a fine powder to be used to prepare the extracts.

2.1.3. Animals

Forty male three-month old albino rats (weighting 150-170 g) were obtained from the breeding unit of the Toxicology and Forensic Medicine Department of the Faculty of Veterinary Medicine at Cairo University. The animals were housed in plastic cages, and were fed a standard laboratory diet and water *ad libitum*. All animal experiments were carried out in accordance with the guide of the care and use of laboratory animals published by the National Institute of Health (NIH Publication No.85-23, revised 1996), and revised by the animals' experiments local ethics committee at Cairo University, Egypt.

2.2. Methods

2.2.1. Tribulus terrestris Extraction

One kg of dried powder of *T. terrestris* was extracted with three liters of the solvent mixture of methanol: acetone: H_2O (2:2:1, respectively), under shaking at 23±1 °C on an orbital shaker (Heidolph Unimax 2010, Germany) for forty-eight hours. The extract was filtered using Whatman No.1 filter paper. The plant residues were re-extracted twice with the same solvent mixture. The pooled filtrates were concentrated under vacuum at 40°C to dryness. The dried crude extract was re-dissolved in methanol for further phytochemical and antioxidant analysis, and was dissolved in distilled water for biological experiment. Unless noted otherwise, all extraction and subsequent characterization experiments were done using three replicates.

2.2.2. Phytochemical Analysis

2.2.2.1. Total Phenolic Content

The total phenolic (TP) content was determined by Folin Ciocalteu reagent assay as described by Singleton and Rossi (1965). A sample aliquot (1 mL) of the *T. terrestris* extract was added to a 25 mL volumetric flask, containing 9 mL of distilled water. One milliliter of Folin Ciocalteu phenol reagent was added to the mixture and shaken. After five minutes 10 mL of 7 % Na_2CO_3 solution were added to the mixture. The solution was diluted to 25 mL with distilled water and mixed using a magnetic stirrer. After incubation for ninety minutes at room temperature, the absorbance was measured at 750 nm with a spectrophotometer (Unicum UV 300, England) against prepared reagent as blank. The total phenolic content was expressed as mg Gallic acid equivalents (GAE)/g dry weight.

2.2.2.2. Total Flavonoid Content

The aluminum chloride method was used for the determination of the total flavonoid (TF) content (Zhishen *et al.*, 1999). One mL of the *T. terrestris* extract was added to a 10 mL volumetric flask, containing 4 mL of distilled water. To the flask, 0.3 mL 5 % $NaNO_2$ was added followed by the addition of 0.3 mL 10 % $AlCl_3$ after five minutes. After six minutes, 2 mL 1M NaOH were added, and the total volume was made up to 10 mL with distilled water. The solution was mixed well and the absorbance was measured against prepared reagent blank at 510 nm using a spectrophotometer (Unicum UV 300, England). The total flavonoid content was expressed as mg quercetin equivalents (QE)/ g dry weight.

2.2.2.3. Identification of Phenolic Acids and Flavonoid Compounds

The dried crude extract of *T. terrestri* (10 mg) was dissolved in 2 mL methanol HPLC spectral grade by vortex mixing for fifteen minutes. The extract was filtrated through a 0.2µm Millipore membrane filter, and was set up to a known volume (2 mL). The phenolic and flavonoid compounds were identified by HPLC (Agilent Technologies 1100 series, Germany), equipped with a quaternary pump (G131A motendel). Separation was achieved on ODS reversed phase column (C18, 25×0.46 cm i.d. 5 µm, Netherlands). The injection volume (35 µL) was carried out with an auto sampling injector. The column temperature was maintained at 35°C. Gradient phenolic compounds' separation was carried out with an aqueous formic acid solution 0.1 % (A) and methanol (B) as a mobile phase at a flow rate of 0.3 mL/min following

the method of Goupy *et al.* (1999). In addition, the flavonoid compounds' separation was carried out with 50 mM H_3PO_4, pH 2.5 (solution A) and acetonitrile (solution B) as a mobile phase at a flow rate of 0.7 mL/min as described by Mattila *et al.* (2000). Elutes were monitored using a UV detector set at 280 nm for the phenolic acids, and at 330 nm for flavonoids. Chromatographic peaks were identified by comparing the retention times with the respective retention times of known standard reference material. Phenolic acids and flavonoid compounds' concentration were calculated by comparing its peak areas with the peak areas of used standards (with known concentration) based on the data analysis of Hewlett Packard software. Phenolic acids and flavonoid compounds were expressed as mg/g dry extract.

2.2.3. Antioxidant Activity

2.2.3.1. DPPH$^{\bullet}$ Radical Scavenging Assay

The DPPH$^{\bullet}$ (2, diphenyl-1-picryl hydrazyl) radical scavenging activity of the *T. terrestris* extract was determined by the method of Chu *et al.* (2000). The DPPH$^{\bullet}$ (0.1 mM) in methyl alcohol was prepared and 0.5 mL of this solution was added to 1 mL of the *T. terrestris* extract at different concentrations (25, 50, 75, 100 µg/mL). The mixture was shaken vigorously, and was allowed to stand at room temperature in the dark for thirty minutes. Butyl hydroxytoluene (BHT, Sigma Aldrich, St. Louis, MO, USA) was used as positive control, whereas the negative control contained the entire reaction reagent minus the extract. Then the absorbance was measured at 515 nm against blank. The capacity to scavenge the DPPH$^{\bullet}$ radical was calculated using the following equation:

DPPH$^{\bullet}$ scavenging effect (Inhibition %) = $((A_c - A_s / A_c) \times 100)$

Where A_c was the absorbance of the control reaction and A_s was the absorbance in the presence of the plant extracts. The results were expressed as IC_{50} (the concentration (mg/mL) of the plant extract that scavenge 50 % of DPPH$^{\bullet}$ radical).

2.2.3.2. Reducing Power

The reducing power was assayed as described by Kuda *et al.* (2005). One mL of the *T. terrestris* extract at different concentrations (25, 50, 75, 100 µg/mL) was mixed with 2.5 mL of phosphate buffer (50 mM, pH 7.0) and 2.5 mL of 1 % potassium ferricyanide. The mixture was then incubated at 50°C for twenty minutes. After the addition of 2.5 mL of trichloroacetic acid (10 %) to the mixture, centrifugation at 3000 rpm for ten minutes was performed. Finally, 1.25 mL from the supernatant was mixed with 1.25 mL of distilled water and 0.25 mL $FeCl_3$ solution (0.1 %, w/v). The absorbance was measured spectrophotometrically at 700 nm. BHT was used as positive control. The results were expressed as EC_{50} (the concentration (mg/mL) of the plant extract that provided the reading of 0.5 absorbance at 700 nm).

2.2.3.3. ABTS$^{\bullet+}$ Antioxidant Assay

ABTS$^{\bullet+}$ assay was carried out according to Arnao *et al.* (2001). Briefly, ABTS$^{\bullet+}$ was dissolved in double distilled water to 7.4 mM concentration, and potassium persulphate was added to a concentration of 2.6 mM. The working solution was then prepared by mixing the two stock solutions in equal quantities. They were allowed to react for 12-16 hours at room temperature in the dark. The solution was then diluted by mixing 1 mL of the ABTS$^{\bullet+}$ solution with 60 mL of methanol to obtain an absorbance of 1.1 ± 0.02 at 734 nm using the spectrophotometer. The *T. terrestris* extracts (150 µL) at different concentration (25, 50, 75, 100 µg/mL) were allowed to react with 2850 µL of the freshly prepared ABTS$^{\bullet+}$ solution for two hours in the dark at room temperature. Then the absorbance was recorded at 734 nm. Trolox was used as a positive control. ABTS$^{\bullet+}$ scavenging activity (%) was calculated using the equation:

ABTS$^{\bullet+}$ scavenging (%) = $((A_0 - A_1) / A_0) \times 100$

Where A_0 is the ABTS$^{\bullet+}$ absorbance of the control (the entire reaction reagent minus the sample), and A_1 is the ABTS$^{\bullet+}$ absorbance in the presence of the sample. The results were expressed as IC_{50} (the concentration (mg/mL) of the plant extract that scavenge 50 % of ABTS$^{\bullet+}$ radical).

2.2.4. Biological Analysis

2.2.4.1. Estimation of Median Lethal Dose 50 (LD_{50})

Twenty mature male rats were divided into four groups (five rats per group) then they were orally administered alpha-cypermethrin in four doses (14, 11.666, 9.722, and 8.101 mg/Kg BW); each group was administered only one dose one time and was monitored for fourteen days. Mortality was assessed and counted in the four groups. The median lethal dose LD_{50} was determined according to the method of Weil (1952). The LD_{50} dose was calculated as 10.65 mg/Kg BW by the end of fourteen days.

2.2.4.2. Experimental Design

Forty mature rats were randomly assigned into four equal groups group1 (Control), rats were kept as control group; group 2 (TT), rats were orally administered (100 mg of *T. terrestris* extract/kg BW); groups 3 (CYP), rats were orally administered 1/20 LD_{50} of alpha-cypermethrin (0.533 mg/kg, BW); and group 4 (TT+CYP), rats were orally administered alpha-cypermethrin at a dose level of 0.533 mg/kg BW along with 100 mg of the *T. terrestris* extract /kg BW. Gastric intubation was used for the administration of both alpha-cypermethrin and the *T. terrestris* extract for sixty-five days.

2.2.4.3. Blood and Tissue Sampling

After the experimental period, blood was withdrawn from the retro-orbital venous plexus of the eye using capillary tubes. It was collected in dry clean tubes, and was centrifuged at 2,000 rpm for ten minutes using cooling centrifuge (Laborzentrifugen, 2k15, Sigma, Germany). Serum was separated and stored at –80°C for biochemical parameters estimations.

The rats were sacrificed by decapitation. Their livers were removed quickly and perfused with pH 7.4 iced phosphate-buffered saline (PBS) to remove blood cells, then blotted on filter paper and frozen at –80°C for biochemical parameters estimation.

Part of the frozen tissues were cut into small pieces and homogenized in 5 mL of cold buffer (0.5 g of Na_2HPO_4 and 0.7 g of NaH_2PO_4 in 500 mL deionized water) (pH 7.4) per gram tissue. After that, they were centrifuged at 4,000 rpm for fifteen minutes at 4°C using a cooling centrifuge, and the supernatant was removed for the

estimation of different biochemical parameters (Hussein *et al.*, 2011).

2.2.4.4. Biochemical Analysis

Serum alanine amino transferase (ALT) and aspartate amino transferase (AST) were determined using commercial kit purchased from Bio Med Diagnostics (Egypt) based on the method of Reitman and Frankel (1957). Liver homogenates were used for the estimation of MDA, GSH and NO levels according to the methods of Watanabe *et al.* (2001), Ellman (1959) and Moshage *et al.* (1995), respectively.

2.2.4.5. Determination of Serum Paraoxonase Activity

The aryl esterase activity of paraoxonase (POX-1) was measured spectrophotometrically in supernatants using phenyl acetate as a substrate. In this assay, aryl esterase/ paraoxonase catalyzes the cleavage of phenyl acetate, resulting in phenol formation. The rate of phenol formation is measured by monitoring the increase in absorbance at 270 nm at 25 ºC. The working reagent consisted of 20 mM of Tris/HCl buffer, pH 8.0, containing 1 mM of $CaCl_2$ and 4 mM of phenyl acetate, as the substrate. The samples diluted in a ratio of 1:3 in buffer were added, and the change in absorbance was recorded following a twenty- second lag time. Absorbance at 270 nm was taken every fifteen seconds for 120 seconds using a UV spectrophotometer (Hussein *et al.*, 2013).

2.2.4.6. Determination of Serum Adiponectin

Serum adiponectin was assayed by an enzyme-linked immunosorbent assay (ELISA) according to Watanabe *et al.* (2006); the kit was supplied by Orgenium Laboratories, Finland.

2.2.4.7. Determination of Serum Tumor Necrosis Factor Alpha (TNF - α)

TNF-α was determined by an enzyme amplified sensitivity immunoassay (EASIA) according to Aukrust *et al.* (1994); the kit was purchased from Biosource, Belgium.

2.2.4.8. Determination of Serum Lipocalin-2

Lipocalin-2 was estimated by ELISA using DRG rats Lipocalin-2 diagnostic kit provided by (DRG International, Inc., USA) according to the manufacturer's instructions.

2.2.5. Statistical Analysis

The data were statistically analyzed using SPSS statistical package, (release 16) for windows. Data were expressed as mean ± SE. Differences between two groups were analyzed by student T test. Multiple comparisons were performed by one-way ANOVA tests.

3. Results

3.1. Total Phenolics and Total Flavonoids

The *T. terrestris* extract showed total phenolic and total flavonoid content of 14.48±0.16 mg GAE/g dry weight and of 3.39±0.08 mg quercetin equivalent/g dry weight, respectively (Table 1).

Table 1. Total phenolic (TP), total flavonoid (TF) and antioxidant activity ($DPPH^{\bullet}$ scavenging, $ABTS^{\bullet+}$ scavenging and reducing powers) of *T. terrestris.*

Sample	TP (mg GAE/g)	TF (mg QE/g)	$DPPH^{\bullet}$ IC_{50} (µg/mL)	$ABTS^{\bullet+}$ IC_{50} (µg/mL)	Reducing power EC_{50} (µg/mL)
T. terrestris	14.48 ± 0.16	3.39 ± 0.08	29.55 ± 0.49	143.30 ± 8.10	115.98 ± 4.81
BHT	-	-	9.78 ± 0.04	-	11.20 ± 0.33
Trolox	-	-	-	16.38 ± 0.43	-

Each assay was carried out in triplicate. Data expressed as mean value ± SD. GAE, gallic acid equivalents. QE, quercetin equivalen. BHT,butylated hydroxytoluene.

3.2. HPLC Profile of Polyphenolic Compounds

The total content of phenolic and flavonoids has been reported previously in *T. terrestris;* however, the characterization of different phenolic and flavonoid compounds is still limited. HPLC screening of the *T. terrestris* extract in the present study revealed the presence of 45 polyphenolic compounds identified as twenty-three phenolic compounds and twenty-two flavonoid compounds (Table 2). In regard to the phenolic compounds, e-vanillic, oleuropen, ellagic, pyrogallol, and vanillic were the leading compounds detected in the highest concentration (28.82, 10.07, 3.12, 2.08, and 1.88 mg/g extract, respectively) of the *T. terrestris* extract in addition to other several phenolic acids, as presented in Table 2.

The major flavonoid compounds (Table 2) detected in the *T. terrestris* extract were hesperidin (73.96 mg/g extract), luteolin 6-arbinose 8-glucose (52.09 mg/g extract), kampferol 3, 2-p-comaroylglucose (19.35 mg/g extract), acacetin (6.65 mg/g extract), quercetrin (4.85 mg/g extract), and apigenin 6-glucose 8-rhamnose (2.10 mg/g extract) in addition to other several flavonoids listed in Table 2.

Table 2. HPLC profile of polyphenolic compounds of *T. terrestris.*

No.	Phenolic compounds	Concentration (mg/g, extract)	Flavonoid compounds	Concentration (mg/g, extract)
1	Pyrogallol	2.08	Luteolin 6-arbinose 8-glucose	52.09
2	Gallic acid	0.16	Luteolin 6-glucose 8-arbinose	1.31
3	4-Amino-Benzoic acid	0.74	Apigenin 6-arbinose 8-glucose	0.91
4	Protocatchuic	0.68	Apigenin 6-rhamnose 8-glucose	1.35
5	Catechein	0.49	Apigenin 6-glucose 8-rhamnose	2.10
6	Catechol	1.26	Luteolin 7-glucose	0.50
7	Epiucatachin	0.66	Narengin	1.08
8	*P*-OH-benzoic	0.93	Hesperidin	73.96
9	Caffeine	0.69	Rutin	1.08
10	Chlorogenic acid	0.46	Apigenin 7-O-neohespiroside	1.09
11	Vanillic acid	1.88	Kampferol 3,7-dirhamoside	1.21
12	Caffeic acid	0.73	Quercetrin	4.85
13	*p*- Coumaric acid	0.25	Rosmarinic	0.22
14	Ferulic acid	0.92	Quercetin	1.75
15	e-Vanillic acid	28.82	Naringenin	1.89
16	Resveratrol	0.55	Kampferol 3,2-*p*-comaroyl glucose	19.35
17	Oleuropen	10.07	Hespirtin	1.04
18	α- Coumaric acid	0.96	Kampferol	0.25
19	Ellagic acid	3.12	Rhamenetin	0.21
20	3,4,5-Methoxy-Cinnamic acid	1.02	Apigenin	0.18
21	Coumarin	0.37	Apigenin-7-glucose	0.18
22	Cinnamic acid	0.98	Acacetin	6.65
23	Salycilic acid	0.38	-	-

3.3. In vitro Antioxidant Activity

Due to the diversity of the antioxidant components in the plant extracts and their mechanism of action, three different assays ($DPPH^{\bullet}$, $ABTS^{\bullet+}$ and reducing power) were used in the present study to assess the *in vitro* antioxidant activity of the *T. terrestris* extract. The $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ radical scavenging activity of the *T. terrestris* extract were compared to BHT and Trolox, respectively, and were expressed as IC_{50} (concentration of the extract where the absorbance of $DPPH^{\bullet}$ or $ABTS^{\bullet+}$ were reduced to 50 % in comparison to absorbance of blank). As presented in Table1, the *T. terrestris* extract possessed $DPPH^{\bullet}$ activity (IC_{50} 29.55 ± 0.49 µg/mL) and $ABTS^{\bullet+}$ activity (IC_{50} 143.30 ± 8.10 µg/mL), compared to BHT and Trolox IC_{50} values (9.78 ± 0.04 and 16.38±0.43µg/mL), respectively. In regard to the reducing power activity, the effective concentration of *T. terrestris* extract that provides the reading 0.5 absorbance at 700 nm (EC_{50}) was found to be 115.98 ± 4.81 µg/mL compared to BHT (11.20 ± 0.33 µg/mL) as presented in Table 1.

3.4. Liver Enzymes

It was observed that treating rats with alpha-cypermethrin (CYP) significantly ($p \leq 0.05$) increased the ALT and AST activity compared to the control group. Whereas, pretreatment with the *T. terrestris* extract (TT) significantly decreased these values in the treated group compared to CYP (Figure 1).

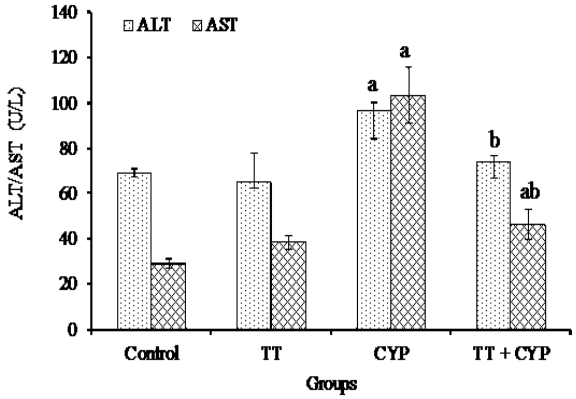

Figure 1. Serum ALT and AST levels in different studied groups.

Data presented as mean ± SE, Number of rats per group n = 10.
[a]Significant difference at $P \leq 0.05$ compared to the control group.
[b] Significant difference at $P \leq 0.05$ compared to the CYP group.

3.5. Oxidant /Antioxidant Parameters

MDA and NO levels were significantly ($p \leq 0.05$) increased in the CYP group when compared to the control group. Whereas, the co-administration of TT significantly alleviated MDA and NO values in the treated group compared to CYP group (Table 3). A significant ($p \leq 0.05$) reduction in POX-1 and GSH was observed in the CYP group compared to the control group. However, the co-administration of TT significantly ($p \leq 0.05$) improved these values in the treated group, making it close to that of the control group (Table 3).

Table 3. Oxidant and antioxidant parameters in different studied groups.

Groups	NO (µmol/g) tissue	POX (IU/g) tissue	GSH (µg/g) tissue	MDA (nmol/g) tissue
Control	4.8±0.3	34.0±1.8	28.6±0.6	119.0±7.8
TT	4.6±0.4	34.6±1.4	29.3±0.3	127.3±3.9
CYP	17.5±0.8[a]	16.5±0.3[a]	17.0±0.8[a]	248.6±14.2[a]
TT + CYP	10.7±0.7[ab]	25.2±2.9[ab]	23.3±0.6[ab]	191.0±5.1[ab]

Data presented as mean ± SE, Number of rats per group n = 10. [a]:Significant difference at $P \leq 0.05$ compared to the control group. [b]:Significant difference at $P \leq 0.05$ compared to the CYP group.

3.6. Inflammatory Markers

The liver inflammation markers (TNF-α, lipocalin, and adiponectin) increased in the CYP group compared to the control group; however, these levels were modulated in the treated group (TT+CYP) to be partially similar to the control compared to the CYP group (Table 4).

Table 4. TNF-α, lipocalin and adiponectin levels in different studied groups.

Groups	TNF-α (Pg/g) tissue	Lipocalin (µg/g) tissue	Adiponectin (ng/g) tissue
Control	0.17±0.008	2.63±0.1	10.2±0.7
TT	0.11±0.04	2.5±0.03	8.6±0.4
CYP	0.34±0.02[a]	4.0±0.2[a]	16.3±0.6[a]
TT + CYP	0.21±0.008[b]	2.9±0.05[b]	12.3±0.6[b]

Data presented as mean ± SE, Number of rats per group n = 10. [a] Significant difference at $P \leq 0.05$ compared to the control group. [b] Significant difference at $P \leq 0.05$ compared to the CYP group.

Person's correlation coefficients were calculated between MDA level and inflammatory markers TNF-α, adiponectin and lipocalin in all groups. There were highly significant positive correlations between the MDA level and the inflammatory markers TNF-α, adiponectin and lipocalin in all groups (p<0.00) (Figure 2).

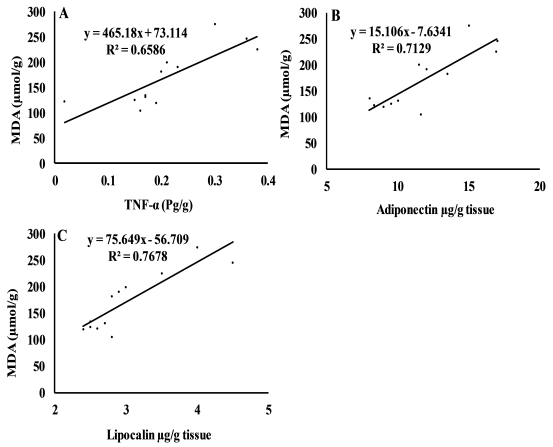

Figure 2. Correlation coefficient between A) MDA and TNFα; B) MDA and adiponectin, and C) MDA and lipocalin.

4. Discussion

Liver diseases represent a critical health problem around the world. In addition to the toxic chemicals such as aflatoxins, high doses of paracetamol and chlorinate hydrocarbons, viral infection; apoptosis, oxidative stress, and inflammation are the main processes that cause the initiation and progression of hepatic disorders (El-Khayat *et al.,* 2013; Miltonprabu *et al.,* 2017). Different herbal medications are used in traditional therapy for their preventive and curative properties against hepatic diseases. Most of these herbal medications have a free radical scavenging effect, and others have variable antioxidant properties (Hussein *et al.,* 2016). In recent years, food and pharmaceutical supplements using new bioactive herbal medications have gained abundant attention for the improvement of hepatic dysfunction (Lin *et al.,* 2014).

The phytochemical analysis of the *T. terrestris* extract in the current study showed an adequate amount of both total phenolic and total flavonoids, in addition to several phenolic and flavonoid compounds which were further screened by HPLC. These results are in accord with previous studies, which reported several phenolic, flavonoids and flavonoid glycosides in *T. terrestris* from different regions (Ivanova *et al.,* 2011; Hammoda *et al.,* 2013).

The *T. terrestris* extract showed antioxidant activity with different mechanisms including free radical scavenging activity in DPPH$^{\bullet}$ and ABTS$^{\bullet+}$ assays, besides the capability to reduce Fe^{3+} to Fe^{2+} in reducing power assay. In this regard, Hammoda *et al.* (2013) reported the antioxidant activities of the *T. terrestris* extract using DPPH$^{\bullet}$ assays, however, Zheleva-Dimitrova *et al.* (2012) evaluated the antioxidant activity of *T. terrestris* containing different commercial herbal preparations from Bulgaria using DPPH$^{\bullet}$, ABTS$^{\bullet+}$ and ferric-reducing antioxidant power. As total polyphenols (a wide class of compounds including phenolic acids, flavonoids, flavonols and anthocyanins) can scavenge free radicals *in vitro* and *in vivo*, the observed antioxidant activities of the *T. terrestris* extract, rich in phenolic and flavonoid compounds in the present study might be attributed to the components of polyphenolic compounds. Sasipriya and Siddhuraju (2015) reported that the antioxidant properties of phenolic compounds can be generally attributed to their redox properties, through different possible mechanisms, such as quenching singlet and triplet oxygen, absorbing and neutralizing free radicals, the transition of metal chelating activity, and/or decomposing peroxides.

The liver is an important organ. Its function is mainly involved in the decomposition and detoxification of toxic compounds, including xenobiotics (pesticides). Subsequently, the permanent alteration in its function causes hepatotoxicity and health implications. Fluctuation of liver enzymes such as ALT and AST are one of symptoms of hepatic damage and liver dysfunction (Rjeibi *et al.,* 2016). The administration of CYP to rats in the present study significantly increased the ALT and AST levels. These results are in accord with the results of Abdou *et al.* (2012). The increase in ALT and AST could be due to the accumulation of CYP in liver tissues, thereby leading to damage in cell membrane; consequently releasing the enzymes in the blood stream. On the other

hand, the pretreatment of rats with TT (TT + CYP group) significantly decreased the levels of ALT and AST enzymes. These results suggest the capability of the TT extract and its polyphenolic bioactive components in the alleviation of liver toxicity induced by CYP through the maintenance of liver cellular membranes' integrity.

CYP increases hepatic lipid peroxidation in rats, and reduces the antioxidative cellular reserves (both the enzymatic and non-enzymatic) leading to a condition of oxidative stress and causing free radical-mediated tissue damage and dysfunction (Sushma and Devasena, 2010).

The current results demonstrated that oxidative stress caused by CYP administration was marked by high oxidative parameters (MDA and NO) and low antioxidant defense parameters (POX-1 and GSH). These findings were in agreement with previous studies that showed an elevation of serum MDA and NO as well as a decrease in the antioxidant enzymes with the CYP exposure (Wang *et al.,* 2009; Hocine *et al.,* 2016).

Pyrethroids-induced oxidative damage may be attributed to their lipophilicity, thus, they can easily penetrate the cell membrane causing membrane lipid peroxidation (Prasanthi and Rajini, 2005); this mechanism could explain the high levels of liver MDA in the current results. The elevation of liver NO in this study was also explained by Wang *et al.* (2009) who indicated that the CYP administration increased inducible NOS (iNOS) and total NOS (T-NOS) concentrations raising the NO level in rats; this oxidative state leads to different organ damages with both biochemical and physiological alterations.

The significant decreases in POX-1 and GSH levels in the current study may be caused by either the inhibition of GSH synthesis or augmented consumption of GSH and POX-1 for scavenging of excess free radicals which cause tissue damage (Raina *et al.,* 2009).

In contrast, the oral administration of the TT extract with CYP in (TT+CYP group) revealed significant increase in liver GSH and POX-1 together with a significant reduction in the liver MDA and NO compared to the CYP group. These results agree with former results of Amin *et al.* (2006) who found a significant increase in liver GSH and a reduction of MDA in diabetic rats treated with TT extract. Also, Lakshmi *et al.* (2012) pointed out that the *T. terrestris* extract administration relieved cadmium toxicity in rats through the restoration of antioxidant, peroxidation and functional indicators in the liver and kidney tissues.

The alleviation effect of the TT extract in the current study could be attributed to the rich presence of flavonoid compounds that have a potential antioxidant activity, such as kaempferol, which causes singlet oxygen scavenging, iron chelation, NADPH oxidase inhibition, and lipid peroxidation chain reaction termination (Fatima *et al.,* 2015).

Quercetin is the most important flavonol generally detected in several natural resources as well as in the *T. terrestris* extract (as observed in Table 2). The Quercetin dose not only act as an antioxidant agent through direct donation of hydrogen atoms and quenching of ROS, but it also induces the intracellular anti-oxidative defense system by the direct interaction with the intracellular signaling cascades related to the antioxidant function (Williams *et al.,* 2004). Similarly, Huang *et al.* (2013) revealed that apigenin, luteolin, and chrysin are all able to attenuate hepatic oxidative stress induced by *tert*-butyl hydroperoxide (tBHP) by means of regulating the gene transcription of hemeoxygenase 1 (HO-1) and glutamate cysteine ligase catalytic (GCLC) and modifier subunit (GCLM) through the activation of extracellular signaling pathways of signal-regulated protein kinase 2 (ERK2), nuclear factor erythroid 2-related factor 2 (Nrf2) nuclear translocation, and nuclear Nrf2–antioxidant responsive element(ARE) in rat primary hepatocytes.

Hepatic oxidative stress stimulates the proliferation of different inflammatory cells which can increase liver deterioration by releasing many cytokines inflammatory markers (Xin *et al.,* 2015). Lipocalin as an inflammatory marker is induced in damaged hepatocytes in deleterious conditions including infection, intoxication, and inflammation in addition to other conditions of oxidative stress (Borkham-Kamphorst *et al.,* 2013). Adiponectin is a surrogate marker for inflammation through the release of proinflammatory cytokines with the special participation of TNF-α (Pennathur and Heinecke, 2007). In the present study, rats intoxicated by CYP showed a significant increase in the hepatic inflammatory markers, namely TNF-α, lipocalin, and adiponectin.

The results of the present study agree with the findings of Salman *et al.* (2010) who reported high plasma adiponectin in chronic liver disease due to the imbalance between its production by adipocytes and metabolism in the liver as a result of the suppression of hepatic catabolism. Moustafa and Hussein (2016) demonstrated that the insecticide pyrethroid increases TNF-α inflammatory gene expression in rats. Also, Raszewski *et al.* (2015) reported that CYP is involved in the production of cytotoxic T cells and activated NK cells, which consequently elevated the TNF-α level. All these studies emphasize the stimulation of liver injury following the CYP administration.

In the current study, the administration of the TT extract in (TT+CYP) group modulated these markers to be partially similar to that of the control group. The effect of the protecting potential of the TT extract through the modulation of TNF-α, lipocalin, and adiponectin levels in rats might be attributed to the bioactive chemical contents of the TT extract specifically the diversity of polyphenolics presented in Table 2. Several studies indicated the involvement of different polyphenolics isolated from natural resources in down-regulation of pro-inflammatory markers including TNF-α, cyclooxygenase-2 (COX-2), iNOS, C-reactive protein (CRP), interleukin-1β (IL-1β), and adiponectin in different experiments concerning liver toxicity (Chtourou *et al.,* 2015).

5. Conclusions

Results of the present study revealed the adequate amount of total phenolic and total flavonoids and several other compounds of phenolic and flavonoids screened for the first time by HPLC in *T. terrestris* growing in Egypt. The *T. terrestris* extract was found to exhibit a potent *in vitro* antioxidant activity using DPPH, ABTS, and reducing power assays. The significant effect of *T. terrestris* extract in the attenuation of hepatotoxicity induced by α-cypermethrin in rats was also investigated

for the first time. The administration of *T. terrestris* extract decreased liver enzymes (ALT and AST), increased liver GSH and POX-1, decreased liver MDA and NO, and decreased hepatic inflammatory markers (TNF-α, lipocalin, and adiponectin). The significant hepatoprotective effect of the *T. terrestris* extract may be attributed to its ability to hunt formed free radicals and induce the antioxidant enzymes expression in addition to the down-regulation of the pro-inflammatory markers that are known to be released following the injury of liver tissues. In summary, these results emphasize the therapeutic potential of the Egyptian *T. terrestris* and suggest its use as a natural hepatoprotective source and health-promoting product.

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Investigating the Antibacterial Potential of Ethanolic and Methanolic Extracts of the *Schinus molle* L Tree

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Abstract

Schinus molle L., an ornamental plant of the Jordanian environment, was acknowledged recently for its therapeutic properties against many microbes. This study aimed at evaluating the antibacterial potential of the ethanolic and methanolic extracts obtained from different parts of the *Schinus molle* L. tree against four strains of bacteria. Data in this study showed that, the ethanolic and methanolic extracts from all experimented plant parts inhibited the growth of *Bacillus subtilis* successfully. However, the best results were obtained from the ethanolic extract of leaves as it resulted in a growth inhibition zone of (22.0 ±0.06 mm), while growth was completely inhibited at MIC value of (1.563 mg/mL). Moreover, growth of *Enterobacter aerogenes* and *Klebsiella pneumoniae* were mostly inhibited after exposure to the methanolic extract of the leaves as it resulted in inhibition zones of (18.0 ±0.086 and 17.0±0.12 mm) respectively, while full growth inhibition was obtained at MIC being (1.563 mg/mL). Moreover, results of disc diffusion assay indicated that *Micrococcus luteus* growth was slightly affected by the leaves' ethanolic extract, while all other types of either ethanolic or methanolic extracts failed to inhibit growth of these bacteria. However, the results of microdilution assay revealed a full growth inhibition of *Micrococcus luteus* when exposed to either fruit or leaves' ethanolic extracts at level of (6.25 mg/mL). However, methanolic extracts from all invistigated plant parts faild to prevent the growth of *Micrococcus luteus*.

Keywords: Antibacterial activity, *Bacillus subtilis*, *Enterobacter aerogenes*, Extract type, *Klebsiella pneumoniae*, *Micrococcus luteus*.

1. Introduction

Synthetic drugs are routinely used as therapeutic agents against microbial diseases. But, recently it has been proven that such compounds might have dangerous or even lethal side effects on humans. Many records have revealed serious numbers of victims of synthetic drugs (Mehani and. Segni, 2013). For example, it was reported that in the United States, 100,000 people are direct victims of synthetic drugs; their deaths were related to the drugs' toxicities (Karimi *et al*., 2015). Also, the high cost of many of these drugs has increased the burden on people especially those who can't even afford the cost of living (Iserin *et al*., 2001; Mehani and. Segni, 2013). In addition to all this, the appearance of new microbial stains with resistant properties against synthetic drugs poses another major threat that highly concerns most scientists in the medical field.

Searching for other new and effective weapons against microbes has been on top of man's priorities for many years. The growing fears regarding the side effects of synthetic drugs on human health have triggered phyto-mining researches to look for other antimicrobial options with minimal hazards on health. Many medicinal plants were acknowledged recently for their remarkable therapeutic properties against many microbes (Jaganthan *et al.*, 2015). Such healing properties of some the plants can be attributed to the production of many active ingredients known as natural products (NP) made by the plant cell machinery (Ochoa-Villarreal *et al.*, 2016). Using medicinal plants for medical purposes was rarely reported to have serious side effects (Hajian, 2013), but in some cases, some remedies of medicinal plants were found to be highly toxic possibly because of the misidentification of these plants, or the incorrect preparation and administration by unprofessional people (Karimi *et al.*, 2015).

Schinus molle L. (also known as American pepper, Peruvian peppertree, escobilla and false pepper) is an evergreen tree which originated in South America, and widely spread to most tropical and subtropical areas in addition to the Mediterranean region (Marongiu *et al*., 2004; Belhamel, *et al.*, 2008). *Schinus molle* L. belongs to the *Anacardiaceae* family and has thin, long leaves, and is mostly distinguished by its bright pink fruits *(Blood, 2001). Schinus molle* L. is commonly grown as an ornamental tree in addition to its usage in the spice production and the beverage industry (Hayouni *et al.*, 2008).

Due to the presence of a high oil content in most of the plant parts that is being rich in monoterpene hydrocarbons and some sesquiterpenes (Bernhard *et al.*, 1983) *Schinus molle* L. has been used in folk medicine (Erazo *et al.*, 2006; Deveci *et al.*, 2010) for the treatment of diuretic, digestive toothache, rheumatism and menstrual pains

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(Daniele *et al.*, 2007). Moreover, it was reported that the extracts of *S. molle* had showed promising activities against several strains of bacteria and fungi (Belhamel, *et al.*, 2008; Rocha *et al*., 2012; Mehani and Segni, 2013). Additionally, (Ibrahim and Al-Naser, 2014) stated that that the oil extracted from the fruit berries of *Schinus moll*e L. showed a promising antifungal potential, as it inhibited the growth of *Botrytis cinerea* fungus. Most of the studies searched the antimicrobial potential of *Schinus moll* L. using essential oils extracted from several plant parts such as the leaves and fruits (berries) after hydrodistillation in a Clevenger-type apparatus (Hayouni *et al*., 2008; Belhamel, *et al*., 2008; Rocha *et al*., 2012; Mehani and Segni, 2013). However, Deveci *et al.* (2010) used hexanic extracts in his experiment against several bacterial strains and oriental cockroach, where the hexanic extracts from the leaves were found to be very promising against *Escherichia coli* and oriental cockroach. In another study, Ibrahim and Al-Naser (2014) used the petroleum ether and n- hexane extracts of *Schinus molle* L. fruits against *Botrytis cinerea* fungus and found that the inhibition of fungi growth varied with the type and dose of the extract. The current study is aimed at evaluating the antibacterial potential of the extracts obtained from different parts of the *Schinus moll*e L. tree (leaves, fruits, Bark and flowers) growing in Jordan against several strains of bacteria using other extraction techniques (namely ethanolic and methanolic extracts).

2. Materials and Methods

2.1. Plant Material and Extract Preparation:

Leaves, ripe fruits, flowers and bark samples were collected directly from a single ten- year old *Schinus molle* L. tree growing on the campus of Jordan University in Amman, Jordan (Latitude: 32° 00' 30.00" N, Longitude: 35° 52' 13.19" E). The leaves and bark samples were collected in May, 2017, whereas the flower samples were collected in July, 2017, and the ripe fruits were collected in November, 2017. The samples were then oven dried at 45°C before being grounded into fine powder. The extraction from each sample was performed by adding either ethanol or methanol to the powder obtained from each plant part with the ratio of 1:10. Next, the extracts were filtered and evaporated to dryness using a rotary evaporator before being dissolved in DMSO to obtain a final concentration of 100 mg/mL.

2.2. Antibacterial Activity

Antibacterial activity of each extract type was evaluated against four bacterial strains (*Bacillus subtilis* ATCC 6633, *Enterobacter aerogenes* ATCC 13048, *Klebsiella pneumoniae* ATCC 31488 and *Micrococcus luteus* ATCC 10240) using Disc diffusion and microdilution assays.

2.2.1. Disc Diffusion Assay:

6mm diameter discs on Muller Hinton agar (Mast Group Ltd. U.K) plates streaked with 100 µL of each bacterial strain suspension were adjusted to 0.5 McFarland (Karlsmose, 2010). Then, 10 mg of each extract type were loaded into the discs. A standard antibiotic (Tetracycline 10 mg/mL) was used as reference. The antibacterial activity was determined by measuring the zone of inhibition after incubation at 37C° for twenty-four hours.

2.2.2. Microdilution Assay

The minimum inhibitory concentration (MIC) was determined using the microdilution method; the extracts were serially diluted according to NCCLS (2000). Ten dilutions were prepared from each extract by being serially diluted (2-fold) with Muller Hinton, potato dextrose broth. Well number eleven consisted of nutrient broth plus Tetracycline (10 mg/mL) and was used as a control. The resulted ten concentrations of the methanolic and ethanolic extracts were from (50) to (0.098) mg/mL. Bacteria inoculate were adjusted to contain approximately 10^5 CFU/mL. The results were visually recorded after incubation in a plate shaker incubator at 37 °C for twenty-four hours.

2.3. Experimental Design

For the disc diffusion assay, the treatments were arranged in a completely randomized design (CRD), where each treatment was replicated five times. The data recorded for the measurement of inhibition zones were analyzed according to the analysis of variance (ANOVA) using (SPSS, version 17) analysis system, while means separation was performed and standard errors (SE) were extracted according to the Tukey's HSD test (Honest Significant Difference) at a probability level of 0.05.

3. Results and Discussion

3.1. Disc Diffusion Assay

The data revealed that the ethanolic extract of all types of the plant material has an antibacterial potential against *Bacillus subtilis* (Table 1, Figure 1). Meanwhile, the antibacterial efficacy of the ethanolic extracts varied significantly according to the type of plant part. The strongest antibacterial activity was obtained from the leaves' extract as it resulted in a maximum inhibition zone of (22.0 mm) which was very close to the results obtained by Tetracycline (24.0 mm) (Table 1). Variation in the antibacterial potential according to the parts of the *Schinus molle* L. tree was also reported by Deveci *et al.* (2010) who showed that the leaves' hexanic extract was more effective against some selected types of bacteria than the fruit hexanic extract. However, according to Deveci *et al.* (2010), the strong antimicrobial activity found in the leaves' extract can be referred to the action of the major chemical components of the leaves extract namely Germacrene D at 20.77 % and Beta-ceryophyllene at 13.48 %. However, these two ingredients were found in very much lower levels in the *Schinus molle* L. fruit extract (Germacrene D: 0.32 % and only traces of Beta-ceryophyllene) (Hayouni *et al*., 2008). This can explain the reason for the superior antibacterial potential of the leaves methanolic and ethanolic extracts as investigated in the current study.

Moreover, in the methanolic extract experiments, all types of the extracts had inhibited the growth of *Bacillus subtilis* (Table 1), while maximum inhibition zones (18.0 and 16.0 mm) were recorded from the fruit and the leaves' extracts compared to the extracts of other plant parts. Tetracycline was significantly the best as it resulted in an

inhibition zone of (22.0 mm) (Table 1). The current results regarding *Bacillus subtilis* were consistent with the results reported by Padin *et al.* (2007) in their study of the efficacy of the ethanolic fruit extract of *Schinus molle* L. of Argentina, recording an inhibition zone of (8.0 mm).

Moreover, the present results showed that only the extracts obtained from either the fruits or leaves have showed antibacterial activity against *Enterobacter aerogenes* in the ethanolic extract experiment resulting in the inhibition zone diameters of (12.0 and 14.0 mm) which were significantly similar to the Tetracycline results (Table 1). On the other hand, the methanolic extract showed antibacterial activity in more parts of *Schinus molle* L. For example, the *Enterobacter aerogenes* growth was hindered when exposed to the extracts of the fruits, bark, or leaves (Table 1), with the maximum inhibition effect obtained from the leaves' methanolic extract (18.0 mm) (Table 1).

Moreover, only the leaves ethanolic extracts had showed antibacterial potential against *Klebsiella pneumoniae* and resulted in an inhibition zone with a diameter of (13.0 mm), while the other plant extracts failed to influence *Klebsiella pneumoniae* (Table 1). On the other hand, fruits and leaves were found to limit the growth of this bacterium by the methalolic extract (Table 1) resulting in a maximum inhibition zone with a diameter of (17.0 mm) (Table 1), even though, this is still significantly lower than the value recorded by Tetracycline (26 mm) (Table 1). However, the current results show that the *Schinus molle* L. leaves ethanolic and methanolic extracts were effective on the *Klebsiella pneumoniae* growth better that what was reported in the findings of Hayouni *et al.* (2008) and Mehani and Segni (2013) who experimented the effectiveness of leaves essential oil of *Schinus moll* L. against *Klebsiella pneumoniae*, and recorded an inhibition zone of (11.0 and 15.0 mm) respectively.

Moreover, the data of Disc diffusion assay showed that *Micrococcus luteus* was slightly affected by the *Schinus molle* L. leaves ethanolic extract, while the ethanolic extracts of the other plant parts did not have any activity against *Micrococcus luteus* (Table 1). On the other hand, all *Schinus molle* L. methanolic extracts failed to ihnibit the growth of this bacterium in the Disc diffusion assay experiment (Table 1).

3.2. Microdilution Assay

Results of the microdilution assay showed that a complete inhibition of *Bacillus subtilis* growth was obtained from the ethanolic extracts of fruits and leaves at MIC value of 1.563 mg/mL, which is similar to the Tetracycline results (Table 2). On the other hand, the fruit methanolic extract was found to be the most effective against *Bacillus subtilis* recording MIC value of 1.563 mg/mL compared to the methanolic extracts of other plant parts (Table 2). Padin *et al*. (2007) studied the effect of the ethanolic extract of *Schinus molle* L. fruits against the growth of *Bacillus subtilis* recording MIC value at 15 mg/mL. However, the current results regarding the fruit ethanolic extract proved to be more effective than what reported by the Padin's *et al*. findings (2007); the current results determined an inhibition zone of (18.0 mm) as well as a less MIC value of 1.563 mg/mL (Tables 1, 2).

Table 1. Diameters of inhibition zones (mm) obtained from disc diffusion assay for ethanolic and methanolic *Schinus molle* extracts against selected strains of bacteria.

Bacillus subtilis				
Ethanolic extract				
Fruit*	Flower	Bark	Leaves	Tetracycline
18.0±0.24 b	7.0±0.07 c	5.0±0.02 c	22.0 ±0.06 ab	24.0 ±0.13 a
Methanolic extract				
Fruit	Flower	Bark	leaves	Tetracycline
18.0±0.05 b	5.0 ±0.06 c	2.0 ±0.03 d	16.0 ±0.07 b	22.0±0.07 a
Enterobacter aerogenes				
Ethanolic extract				
Fruit	Flower	Bark	leaves	Tetracycline
12.0 ±0.06 a	-	-	15.0 ±0.17 a	20.0 ±0.46 a
Methanolic extract				
Fruit	Flower	Bark	leaves	Tetracycline
15.0 ±0.16 b	-	6.0 ±0.07 c	18.0 ±0.086 b	22.0 ±0.10 a
Klebsiella pneumoniae				
Ethanolic extract				
Fruit	Flower	Bark	leaves	Tetracycline
-	-	-	13.0 ±0.067 b	20.0 ±0.32 a
Methanolic extract				
Fruit	Flower	Bark	leaves	Tetracycline
6.0 ±0.03 c	-	-	17.0 ±0.12 b	26.0 ±0.25 a
Micrococcus luteus				
Ethanolic extract				
Fruit	Flower	Bark	leaves	Tetracycline
-	-	-	2.0 ±0.05 b	20.0 ±0.12 a
Methanolic extract				
Fruit	Flower	Bark	leaves	Tetracycline
-	-	-	-	12.0 ±0.08 a

*Each value represents mean ± standard error (SE) of five replicates. Means within each raw were analyzed separately, and values with different letters are significantly different according to Tukey HSD at $P<0.05$.

Also, the MIC values of *Enterobacter aerogenes* revealed that growth was totally inhibited when treated with fruit, bark or leaves' ethanolic extracts. The best results were obtained from the fruit and leaves' extract at MIC value of 3.125 mg/mL; however, Tetracycline was most efficient against *Enterobacter aerogenes* at MIC value of (1.563 mg/mL) (Table 2). On other hand, the methanolic extracts from all studied plant parts were found to possess antibacterial activities against *Enterobacter aerogenes*, while extract from leaves was the most promising as it resulted in full inhibition of *Enterobacter aerogenes* at MIC level of (1.563 mg/mL), which is similar to Tetracycline results (Table 2). The antibacterial potential of other *Schinus* species against *Enterobacter aerogenes* was also experimented by few researchers, for example Gehrke *et al.* (2013) reported a promising antibacterial potential of the methanolic extract of *Schinus lentiscifolius* aerial parts against this bacterium at MIC value of (100 µg/mL).

Table 2. Minimal inhibition concentration (MIC) values (mg/mL) of ethanolic and methanolic *Schinus molle* extracts against selected strains of bacteria.

Bacillus subtilis				
Ethanolic extract (mg/mL)				
Fruit	Flower	Bark	leaves	Tetracycline
1.563	3.125	6. 25	1.563	1.563
Methanolic extract (mg/mL)				
Fruit	flower	Bark	leaves	Tetracycline
1.563	6. 25	-	3.125	1.563
Enterobacter aerogenes				
Ethanolic extract (mg/mL)				
Fruit	Flower	Bark	leaves	Tetracycline
3.125	-	6. 25	3.125	1.563
Methanolic extract (mg/mL)				
Fruit	Flower	Bark	leaves	Tetracycline
3.125	6.25	6.25	1.563	1.563
Klebsiella pneumoniae				
Ethanolic extract (mg/mL)				
Fruit	Flower	Bark	leaves	Tetracycline
-	-	-	3.125	1.563
Methanolic extract (mg/mL)				
Fruit	Flower	Bark	leaves	Tetracycline
-	-	-	1.563	1.563
Micrococcus luteus				
Ethanolic extract (mg/mL)				
Fruit	Flower	Bark	leaves	Tetracycline
6. 25	-	-	6. 25	1.563
Methanolic extract (mg/mL)				
Fruit	flower	Bark	leaves	Tetracycline
-	-	-	-	3.125

Moreover, according to the current results, only leaves' ethanolic extract was able to inhibit growth of *Klebsiella pneumoniae* at MIC of (3.125 mg/mL) (Table 2), which contrasted with Padin *et al.* (2007) findings, as they reported that only fruit ethanolic extract against this bacterium was effective at MIC of (14 mg/mL). The results of this study indicated that leaves' methanolic extract was found to be very promising at MIC value of (1.563 mg/mL) which is similar to the MIC value of the Tetracycline treatments (Table 2). On the other hand, Hayouni *et al.* (2008) applied essential oil extracted from the leaves of *Schinus moll* L. against *Klebsiella pneumoniae* and found it to be effective only at MIC levels that exceeded 72 mg/mL. This indicates that, methanolic and ethanolic extracts of leaves were more effective against this bacterium than leaves' essential oil as proven in the present study.

Additionally, the current results showed that growth of *Micrococcus luteus* was totally inhibited when exposed to either fruit or leaves' extracts at level of (6.25 mg/mL), whereas growth was totally inhibited at the Tetracycline level of (1.563 mg/mL) (Table 2). Moreover, the methanolic extracts from all the invistigated plant parts faild to prevent the growth of *Micrococcus luteus* (Table 2). In another related study, Deveci *et al*. (2010) reported a succesfful inhibition of *Micrococcus luteus* growth after exposure to hexanic extracts of either leaves or fruits of *Schinus molle* L. at MIC values of (1 and 2 mg/mL) respectively.

It was noted from the current data that the antibacterial potential has also varied with the type of the extacting solvent (methaol or ethanol), (Tables 1, 2). Methanol was reported to be more commonly used as an extacting solvent than ethanol, because it is more polar (0.762) (ethanol relative polarity is 0.654), which allows methanol to dissolve more polar compounds, in addition to the fact that methanol is less expensive, relatively easily-evaporated, and is free of regulation compared to ethanol, (Eloff, 1998). Also, in another related study, it was found that the differences in chemical composition, and the availability of the extractable compund in each plant part can contribute to the diffirence in the antioxidant potential between the methaolic and ethanolic extracts (Sultana *et al.* 2007).

Conclusions

The obtained results revealed presence of antibacterial activity in *Schinus molle* L. tree against the tested bacterial strains. Moreover, data showed a variation in the antibacterial potential of this tree against each tested bacterial strain according to plant part and type of extract. The ethanolic extract of leaves was the best to cause growth inhibition of *Bacillus subtilis*, while the growth of *Enterobacter aerogenes* and *Klebsiella pneumoniae* were mostly inhibited after exposure to the methanolic extract of leaves. Moreover, the results of Disc diffusion assay indicated that *Micrococcus luteus* growth was slightly affected by the leaves ethanolic extract, while all other types of either ethanolic or methanolic extracts failed to inhibit the growth of this bacterium.

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Morphometric and Meristic Characteristics of *Salmostoma bacaila* (Hamilton, 1822) (Cyprinidae) from the Ganges River in Northwestern Bangladesh

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Abstract

This study is performed for the determination of morphometric characters including length-weight relationships (LWRs) and length-length relationships (LLRs) using 9 linear dimensions and meristic characteristics covering various fin-rays of *Salmostoma bacaila* (Hamilton, 1822) from the Ganges River in northwestern (NW) Bangladesh. A total of 236 individuals of *S. bacaila* were collected occasionally from the Ganges River from July 2015 to June 2016, using traditional fishing gears including a cast net (mesh size ranges: 1.5 - 2.5 cm), a gill net (mesh size ranges: 1.5–2.0 cm), and a square lift net (mesh size: ~2.0 cm). For each individual, a total of nine various lengths were measured nearest to 0.01 cm with digital slide calipers, and the body weight was measured to the nearest 0.01g accuracy by an electronic balance. The LWRs were calculated using the formula: $W = a \times L^b$, where W is the body weight (g), L is the length (cm), and *a* and *b* are LWRs parameters. Fin-ray numbers from all fins as well as scales were computed by a magnifying glass. Total length (TL) varied from 5.9 -11.5 cm and the total body weight (BW) ranged from 1.31-8.8g. All LWRs were highly significant ($p < 0.001$) with r^2 values ≤ 0.959. Based on r^2 value, LWR by BW *vs.* TL ($W= a \times L^b$) was the best fitted model among nine equations. In addition, the LLRs were also significant with r^2 values ≤ 0.985. According to r^2 value, LLR by TL *vs.* FL (TL = $a + b\times$ FL) was the best fitted model among eight equations. The fin formula of *S. bacaila* is: dorsal, D. 8-9 (2–3/6-7); pectoral, Pc. 12 (2-4/8-10); pelvic, Pv. 8-9 (2–4/8-10); anal, An. 13-16 (2-4/10-13); caudal, Ca. 20–24 (6–7/14–17), respectively. This study would be very operative for species identification and stock assessment in the Ganges River of NW Bangladesh and the contiguous ecosystems.

Keywords: *Salmostoma bacaila*, Morphometric, Meristic, Fin rays and scale, Ganges River, Bangladesh

1. Introduction

The Large razorbelly minnow, *Salmostoma bacaila* (Hamilton, 1822), is a small indigenous freshwater cyprinid occurring in rivers, ponds, *beel*s and inundated fields throughout the Indian sub-continent including Bangladesh, India, Pakistan, Nepal, and also in Afghanistan (Froese and Pauly, 2016). It has a high nutritional value, containing excellent amounts of iron, zinc and vitamin-A (Thilsted *et al.*, 1997 Mohanty *et al.*, 2013). This fish species is listed in the IUCN as least concern in Bangladesh (IUCN Bangladesh, 2015) and worldwide (IUCN 2016).

Morphometric and meristic traits are very helpful for the identification and classification of any fish species in a laboratory or in the fields (Bagenal and Tesch, 1978; Jayaram, 1999; Nawer *et al.*, 2017). Additionally, morphometric characters have a significant role in fisheries research as it is used for comparing life history and the morphological traits of the populations of different regions (Hossain, 2010; Hossen *et al.*, 2018). To the best of the authors' knowledge, a few studies were conducted on *S. bacaila* including length-weight and length-length relationships (Masud and Singh, 2015; Islam and Mia, 2016; Muhammad *et al*., 2016; Baitha *et al.*, 2017; Nath *et al.*, 2017). However, none of these studies covered the morphometric and meristic using multi-linear dimensions from the Ganges River. Therefore, the objectives of the present study are to describe the morphometric and meristic characteristics of *S. bacaila* in the lower part of Ganges River in NW Bangladesh using multi-linear dimensions.

2. Material and Methods

This study was performed in different parts of the Ganges River, known also as the Padma River in Bangladesh (Charghat: 24°15' N, 88°44' E; and Shaheb Bazaar: 24°20' N, 88°34'). A total of 236 specimens of *S. bacaila* were caught using different fishing gears including a cast net (mesh size ranges: 1.5 - 2.5 cm), a gill net (mesh

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size ranges: 1.5–2.0 cm), and a square lift net (mesh size: ~2.0 cm) during July 2015 to June 2016. The fresh samples were immediately iced on site, and were preserved after reaching the laboratory in 10 % formalin for further use in this study. Fin-ray numbers from all fins were counted using a magnifying glass. Preserved specimens were individually weighed by an electronic balance (Shimadzu, EB-430DW; Shimadzu Seisakusho, Tokyo, Japan) with 0.1 g accuracy and different linear dimensions i.e., lengths (see Table 1 and Figure 1) were measured to the nearest 0.1cm by digital slide calipers.

The equation: $W= a\times L^b$ was used to calculate the LWR, where W is the body weight (g), and L is the nine different lengths in cm. Linear regression analyses based on natural logarithms: ln (W) = ln (a) + b ln (L) were used to estimate the regression parameters a and b. In addition, 95% confidence intervals (CL) of a and b, and the co-efficient of determination (r^2) were estimated. Outliers that seemed extremes were deleted from the regression analyses according to Froese (2006). To verify whether b values obtained in the linear regressions were significantly different from the isometric value (b = 3), a t-test was done (Sokal and Rohlf, 1987). The LLRs (eight relationships) were estimated by linear regression analysis (Hossain *et al.*, 2006). For statistical analysis, GraphPad Prism 6.5 Software was used. All statistical analyses were considered significant at the level of 5% ($p< 0.05$).

3. Results

Table 1 demonstrates the descriptive statistics for the length and weight measurements of *S. bacaila* in the Ganges River of NW Bangladesh. The total sample (n), regression parameters and 95 % confidence intervals for a and b of the LWRs, coefficients of determination (r^2) and growth type of *S. bacaila* were given in Table 2. The calculated allometric coefficient (b) indicated isometric growth (b = 3.00). The LWRs were highly significant ($p<0.001$), with all r^2 values ≤ 0.959.

Based on r^2 value, LWR by BW *vs.* TL was the best fitted model among nine equations. Moreover, the LLRs (eight relationships) along with the estimated parameters and the coefficient of determination (r^2) were given in Table 3. Also, the calculated LLRs were highly significant ($p<0.001$) with r^2 values ranging from ≤ 0.985. According to r^2 value, LLR by TL *vs.* FL was the best fitted model among eight equations. All the meristic characteristics were given in Table 4.

Table 1. Morphometric measurements of the *Salmostoma bacaila* (n = 236) captured from the Ganges River, northwestern Bangladesh.

Measurements	Min (cm)	Max (cm)	Mean ± SD	95% CL	%TL
TL (Total length)	5.90	11.50	8.16 ± 0.88	8.05 - 8.28	
BW (Body weight)	1.31*	8.80*	3.31 ± 1.22	3.16 - 3.47	
FL (Fork length)	5.50	10.30	7.23 ± 0.79	7.13 - 7.33	88.55
SL (Standard length)	4.80	9.20	6.58 ± 0.72	6.49 - 6.67	80.61
PrDL(Pre-dorsal length)	3.00	6.20	4.46 ± 0.48	4.40 - 4.53	54.71
PoDL(Post-dorsal length)	3.50	6.70	4.93 ± 0.52	4.87 - 4.99	60.42
PvL (Pelvic length)	2.20	4.70	3.37 ± 0.37	3.32 - 3.42	41.31
AnsL(Anus length)	3.00	6.30	4.56 ± 0.49	4.50 - 4.62	55.89
PrAnL(Pre-anal length)	3.10	6.40	4.66 ± 0.50	4.60 - 4.72	57.10
PoAnL(Post-anal length)	3.80	7.60	5.49 ± 0.59	5.41 - 5.56	67.26

Min, minimum; Max, maximum; SD, standard deviation; CL, confidence limit for mean values; TL, total length; SL, standard length; FL, fork length; BW, body weight; PrDL, pre-dorsal length; PoDL, post-dorsal length; PvL, pelvic length; AnsL, anus length; PrAnL, pre-anal length; PoAnL, post-anal length; *, weight in g.

Table 2. Descriptive statistics and estimated parameters of the length-weight relationships of *Salmostoma bacaila* (n = 236) from the Ganges River, northwestern Bangladesh.

Equation	Regression parameters		95% CL of a	95% CL of b	r^2
	a	b			
BW= $a \times TL^b$	0.0051	3.06	0.0043 - 0.0061	2.98 - 3.14	0.959
BW= $a \times FL^b$	0.0079	3.04	0.0067 - 0.0093	2.95 - 3.12	0.958
BW= $a \times SL^b$	0.0108	3.02	0.0091 - 0.0128	2.93 - 3.11	0.948
BW= $a \times PrDL^b$	0.0379	2.96	0.0328 - 0.0438	2.87 - 3.06	0.941
BW= $a \times PoDL^b$	0.0227	3.10	0.0192 - 0.0286	2.99 - 3.20	0.936
BW= $a \times PvL^b$	0.0938	2.90	0.0812 - 0.1084	2.78 - 3.02	0.908
BW= $a \times AnsL^b$	0.0353	2.97	0.0302 - 0.0411	2.87 - 3.07	0.934
BW= $a \times PrAnL^b$	0.0310	3.01	0.0264 - 0.0364	2.91 - 3.12	0.932
BW= $a \times PoAnL^b$	0.0188	3.01	0.0159 - 0.0222	2.92 - 3.11	0.940

n, sample size; a and b are regression parameters; CL, confidence intervals for mean values; r^2, co-efficient of determination

Table 3. The estimated parameters of the length-length relationships ($y = a + b \times x$) of *Salmostoma bacaila* (n=236) from the Ganges River, northwestern Bangladesh.

Equation	Regression parameters		95% CL of *a*	95% CL of *b*	r^2
	a	*b*			
TL = *a* + *b*× FL	0.1590	1.11	0.0183 to 0.2997	1.09 - 1.13	0.982
TL = *a* + *b*× SL	0.1094	1.22	-0.0197 to 0.2384	1.21 - 1.24	0.985
TL = *a* + *b*×PrDL	0.2442	1.77	0.0235 to 0.4649	1.73 - 1.82	0.956
TL = *a* + *b*×PoDL	-0.1311	1.68	-0.3610 to 0.0989	1.64 - 1.73	0.956
TL = *a* + *b*×PvL	0.3724	2.31	0.0889 to 0.6560	2.23 - 2.40	0.927
TL = *a* + *b*×AnsL	0.1971	1.75	-0.0022 to 0.3963	1.70 - 1.79	0.964
TL = *a* + *b*×PrAnL	0.0824	1.73	-0.1204 to 0.2852	1.69 - 1.78	0.964
TL = *a* + *b*×PoAnL	0.1218	1.47	-0.0905 to 0.3340	1.43 - 1.50	0.960

TL, total length; SL, standard length; FL, fork length; PrDL, pre-dorsal length; PoDL, post-dorsal length; PvL, pelvic length; AnsL, anus length; PrAnL, pre-anal length; PoAnL, post-anal length; *a*, intercept; *b*, slope; CL, confidence limit for mean values; r^2, co-efficient of determination

Table 4. Meristic counts of *Salmostoma bacaila* (n=236) from the Ganges River, northwestern Bangladesh.

Meristic data	Numbers	Unbranched	Branched
Dorsal fin rays	8-9	2-3	6-7
Pectoral fin rays	12	2-4	8-10
Pelvic fin rays	8-9	2-3	5-7
Anal fin rays	13-16	2-4	10-13
Caudal fin rays	20-24	6-7	14-17

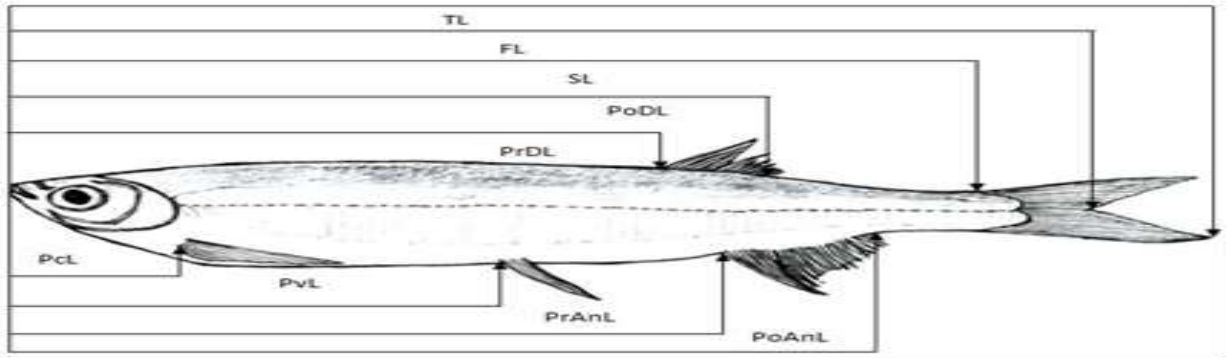

Figure 1. Showing the morphometric measurement of *Salmostoma bacaila* from the Ganges River, northwestern Bangladesh.

4. Discussion

Information on the morphometric and meristic traits are quite scant for *S. bacaila* in the Ganges River, NW Bangladesh. Some works on length-length and length-weight relationship of *S. bacaila* have been done in Bangladesh by Islam and Mia (2016), and in India by Masud and Singh (2015), and Nath *et al*. (2017). In this study, the maximum length was found to be 11.5 cm TL in the Ganges River, which is higher than that in the Atrai River, Dinajpur, Bangladesh (TL= 10.5 cm; Islam and Mia, 2016) and that in the Barak River, Assam, India (TL= 10.4 cm; Nath *et al.*, 2017), but it is lower than the following values: FishBase value 18.0 cm TL (Menon, 1999), 15.2 cm TL from the river Yamuna, India (Masud and Singh, 2015), 15.7 cm TL from Gandak River, Bihar, India (Baitha *et al.*, 2017), and 16.3 cm TL from the Indus River, Pakistan (Muhammad *et al.*, 2016). However, declining in the maximum sizes of *S. bacaila* in the Ganges River might be attributed either to the absence of larger-sized individuals in the populations of fishing grounds (Khatun *et al*., 2018), or simply because fishermen did not go where the larger size might exist. The evidence on maximum length/size is quite helpful to estimate the asymptotic length and growth coefficient of the fishes, which are essential for fisheries stock assessment and management (Hossain *et al*., 2016a, b, 2017).

In the present study, the regression parameter *b* value was found to be 3.06 which exhibits the isometric growth pattern. However, negative allometric growth was reported from several habitats including the Atrai River in Bangladesh (b = 2.76) by Islam and Mia (2016), the river Yamuna in India (b = 2.86) by Masud and Singh (2015), the Barak River in Assam, India (b = 2.47) by Nath *et al.* (2017), the Gandak River in Bihar, India (b = 2.80) by Baitha *et al.* (2017), and the Indus River in Pakistan (b=2.88) by Muhammad *et al.* (2016). The allometric co-efficient (b) values of LWRs ranging from 2.5 to 3.5 are more common (Froese, 2006). In the present study, most of the *b* values were within the limit (2.5–3.5). However, the *b* values may vary in the same species because of the combination of various factors including habitat, stage of stomach fullness, seasonal effect, gonadal maturation, gender, physiology, preservation methods and differences in the observed length ranges of the specimens collected (Hossen *et al.*, 2016), which are not accounted in this

study. In addition, all the LLRs were highly correlated (Table 3) ($p < 0.001$). Furthermore, the fin formula for *S. bacaila* is: dorsal, D. 8-9 (2–3/6-7); pectoral, Pc. 12 (2-4/8-10); pelvic, Pv. 8-9 (2–4/8-10); anal, An. 13-16 (2-4/10-13); caudal, Ca. 20–24 (6–7/14–17). However, in an earlier study, Rahman (2005) reported the fin formula of *S. bacaila* as D. 10(2/8); P_1. 12-13; P_2. 9; A. 14-15(2/12-13), which is quite similar with the current study.

The present study serves as a priceless tool for the fishery manager to recognize *S. bacaila* in the laboratory or fields and commence stock assessment of the existing stock of this species in the Ganges River, NW Bangladesh and other subtropical countries. Also, these results update the information of the FishBase, and provide an important baseline for future studies within the Ganges River and the adjacent ecosystems.

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Conflicts of interest

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

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Seroprevalence of *Toxoplasma gondii* in Cancer Patients Admitted to Hospitals of the Royal Medical Services in Jordan

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Abstract

Several seroprevalence studies on *Toxoplasma gondii* were conducted in Jordan, but none of them investigated its seroprevalence among patients who suffer from different types of cancer. Thus, the present cross-sectional study on two-hundred Jordanian cancer patients and ninety healthy normal subjects (controls) was conducted in order to detect the presence of anti-*T. gondii* antibodies (IgG and IgM) serologically using commercial ELISA. Out of the two-hundred recruited cancer patients, anti-*T. gondii* IgG and IgM antibodies were detected in seventy-nine and five patients (39.5 % and 2.5 %) respectively, and four patients (2 %) had both IgG and IgM antibodies. In the control group, eleven cases (12.2 %) and only one case (1.1 %) were tested positive for IgG and IgM antibodies respectively. In addition, only one case from the control group (1.1 %) had both IgG and IgM antibodies against *T. gondii*. The difference in the IgG seropositivity rates between the patients and the control group is statistically significant (P <0.05) indicating that these patients are at a higher risk to acquire toxoplasmosis. The seroprevalence of *T. gondii* IgG and IgM antibodies increased significantly with age in the control group only. More studies are required in order to investigate the possibility of dissemination, and the severity of toxoplasmosis among cancer patients especially the IgM-positive patients which indicate a current infection.

Keywords: Toxoplasmosis, Seroprevalence, Cancer, ELISA, IgG, IgM, Chemotherapy, Jordan.

1. Introduction

Toxoplasma gondii is a single-cell apicomplexan parasite which infects mammals and birds worldwide causing toxoplasmosis (Robert-Gangneux and Darde, 2012; Fallahi *et al.*, 2018). Infection with *T. gondii* starts upon the ingestion of the infective stage (sporulated oocysts) with contaminated foods or drinks, often shed with the feces of infected cats. Moreover, toxoplasmosis can be acquired upon the ingestion of raw/undercooked infected mammalian or bird meat containing tissue cysts (Cong *et al.*, 2015).

Toxoplasmosis is highly prevalent in humans worldwide (affecting approximately 30- 50 % of the world population) especially among young adults (Furtado *et al.*, 2011, Flegr *et al.*, 2014). The seroprevalence of toxoplasmosis appears to vary from region to region depending on the exposure to the infective stage (the oocysts) (Furtado *et al.*, 2011). While it can reach up to 47 % in France and 14 % in the United States of America, the highest prevalence of toxoplasmosis was reported to occur in South America where its seropositivity can rise up to more than 80 % (Furtado *et al.*, 2011). In the Middle East and other developing countries, there are no accurate statistics for the prevalence of toxoplasmosis. In Jordan, toxoplasmosis IgG antibodies were detected in 66.5 % in a study performed on undergraduate university females (Obaidat *et al.*, 2015). Furthermore, upon examining the prevalence of toxoplasmosis among Jordanian pregnant women, it was found that it reached up to 66.9%, therefore, detecting *T. gondii* antibodies is recommended especially in women who are reported to have multiple miscarriages/abortions (Jumaian, 2005).

In several previous studies, *T. gondii* was reported as the primary infectious agent of congenital infection. However, recent studies confirmed the clinical importance of *T. gondii* and the importance of the infection reactivation among immunocompromised patients (Robert-Gangneux and Darde, 2012). In immunocompetent individuals toxoplasmosis is usually asymptomatic in 90 % of the cases, while the remaining symptomatic cases exhibit flu-like symptoms, ocular disease or cervical lymphadenopathy (Montoya and Liesenfeld, 2004). Toxoplasmosis is severe and could be fatal for some immunocompromised patients, such as those with acquired immune deficiency syndrome (AIDS) (Fallahi *et al.*, 2018).

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Several previous studies suggested that *T. gondii* is an important opportunistic pathogen in immunocompromised patients. A recent review conducted a global meta-analysis and assessed the prevalence of *T. gondii* infection in immunocompromised individuals upon observing the electronic databases for *T. gondii* infection in HIV/AIDS patients, cancer patients, and transplant recipients in a total of seventy-two eligible studies. In the same review, the estimated pooled prevalence of *T. gondii* infection in cancer patients and the control was 26.0 and 12.1% respectively (Wang *et al*., 2017).

When *T. gondii* affects the host, it is usually transmitted through the gastrointestinal (GI) tract to the body in the tachyzoite form, which in turn will invade various parts of the body (mainly the brain, the muscles and the heart) (Jumaian, 2005). In healthy individuals, *T. gondii* enters a latent/ dormancy phase providing a lifelong immunity against this parasite, but the multiplication of bradyzoite may cause destruction for the tissues forming lesions (Flegr *et al*., 2014). However, if the immunity of the individual is weakened by a disease (HIV, cancer, etc.), or by taking certain medications such as corticosteroids, chemotherapy, radiotherapy, *T. gondii* inactive bradyzoites can be reactivated again and transformed into a tachyzoite stage where they can start an acute infection by attacking the surrounding tissue (Fallahi *et al.*, 2018).

In the present study, the main aim was to investigate the seropositivity for *T. gondii* antibodies (IgG and IgM) in cancer patients. The interest in these patients can be attributed to the fact that cancer patients are considered to be immunocompromised due to the nature of their immunosuppressive therapy and as a result, they are expected to be at a higher risk for acquiring toxoplasmosis (new infection or reactivation from previous infection) compared to controls.

2. Materials and Methods

2.1. Study Design and Recruited Patients

This is a cross-sectional study conducted in the hospitals of the Royal Medical Services from November, 2016 until April, 2017 in order to investigate the presence of anti-*T. gondii* antibodies in cancer patients, and to compare them with healthy normal individuals. The ethical approval to conduct the present study was taken from the Hashemite University Institutional Review Board. Also, each participant signed a consent form individually before blood collection. The study recruited two-hundred cancer patients and ninety healthy individuals as a control. Demographic data were collected from patients and controls using a structured questionnaire where the clinical information for each patient was retrieved from the medical report of each patient.

2.2. Serological Assay (ELISA) for the Detection of Anti-Toxoplasma IgG and IgM Antibodies:

Three to five ml of venous blood were collected from each patient and control individually in plain tubes under sterile conditions. The sera were separated, and preserved at -20 °C until being examined. The serological technique, ELISA, was used for the determination of anti-*T. gondii* IgG and IgM antibodies in the serum of the recruited cancer patients and controls. The ELISA kit was provided by a commercial manufacturer (NovaTec®, Germany). The manufacturer instructions were followed for the serum samples and controls and the researchers employed the manufacturer cut-off value of 10 IU/mL of anti-*Toxoplasma* antibody (IgG and IgM) to differentiate between positive (latent, pre-existing or current infection) and negative values.

2.3. Statistical Analysis

Statistical analyses were performed using SPSS statistical computer software for WINDOWS (version 20, IBM Inc., USA). Statistical software was used for the calculation of the seroprevalence of toxoplasmosis among cancer patients and its correlation with variables such as age and sex. The Chi-square test was used for group comparisons (cancer versus normal). Statistical significance was set at 5 %, and all the associations that showed a $P < 0.05$ were considered significant.

3. Results

Using ELISA method, IgG antibodies to *T. gondii* were found in seventy-nine cancer patients (39.5 %) and in eleven of the healthy controls (12.2 %). However, IgM antibodies were found in five cancer patients (2.5 %) and in only one healthy control (1.11 %) (Table 1). The difference in the IgG seropositivity between the patient and the control group is statistically significant ($P < 0.05$). In contrast, the difference in IgM seropositivity between cancer patient and the control group was not statistically significant. The seropositive rates of anti-*T. gondii* IgG were higher in lung, uterine, thyroid and lymph node cancers (50 %), and the lowest rates were observed in stomach and gastric cancer (16.7 %). No anti-*T. gondii* IgG and IgM were detected in the blood of patients with esophageal, bronchial, chest wall mass and pelvic cancers. The seropositive rates of anti-*T. gondii* IgM was observed only in patients with colon and bone cancer, and their ratios were as follow: 17.7 % and 66.7 % respectively. The results were not statistically significant ($P < 0.05$) (Table 2). The seropositivity of anti-*T. gondii* IgG and IgM among the different age groups of cancer patients are shown in Table 3. The highest seropositivity was observed in the age group of 41-50 years in both patients and controls (45.2 % and 66.7 % respectively), (Table 3). The seropositivity in males and females were 42.3 % and 38 %, in cancer patients and 14.3 % and 11.6 % in the controls, respectively. There was no statistical difference in the seropositivity of anti-*T. gondii* IgG and IgM among males and females (Table 3).

Table 1. Seroprevalence of *Toxoplasma gondii* IgG and IgM antibodies in cancer patients and control groups

Antibody	Cancer patients (n = 200)		Healthy controls (n = 90)		*P*- value
	No.	%	No.	%	
Anti-*T.gondii* IgG	79	39.5	11	12.2	< 0.05*
Anti-*T.gondii* IgM	5	2.5	1	1.1	0.44

* Results of Chi-square tests by *P* value of <0.05 as significant difference

Table 2. The seropositivity rates of anti-*T. gondii* IgG and IgM in the cancer patient according to the type of cancer

Cancer type	No. patients	IgG-positive		IgM-positive	
		No.	%	No.	%
Breast cancer	65	28	43.1	0	0.0
Leukemia	20	8	40.0	0	0.0
Lymphoma	18	5	27.8	0	0.0
Colon cancer	17	7	41.2	3	17.7
Myelodyplastic syndrome (MDS)	10	4	40.0	0	0.0
Lung cancer	8	4	50.0	0	0.0
Uterine cancer	8	4	50.0	0	0.0
Stomach and gastric cancer	6	1	16.7	0	0.0
Rectal cancer	6	2	33.0	0	0.0
Myeloma	4	1	25.0	0	0.0
Liver cancer	3	1	…	0	…
Bone cancer	3	1	…	2	…
Esophageal cancer	3	0	…	0	…
Pancreatic cancer	3	1	…	0	…
Bronchial cancer	2	0	…	0	…
Thyroid cancer	2	1	…	0	…
Chest wall mass cancer	2	0	…	0	…
Pelvic cancer	2	0	…	0	…
Lymph node cancer	2	1	…	0	…
Others	16	10	62.5	0	0.0

Seropositivity rates were not shown when the total number of patients was less than 3.

Table 3. *Toxoplasma gondii* IgG seropositivity in cancer patients and controls according to age and gender.

	Cancer patients (n =200)		*Healthy controls (n=90)*	
Factors	Total No. (Positive %)	*P*- value	Total No. (Positive %)	*P*- value
Age (years)				
<20	6 (16.7)		6 (0)	
21-30	11 (18.2)		35 (11.4)	
31-40	28 (39.3)	0.35	27 (3.70)	*<0.05
41-50	42 (45.2)		3 (66.7)	
>51	113 (40.7)		19 (21.1)	
Total	200 (39.5)		90 (12.22)	
Gender				
Male	71 (42.3)		21 (14.3)	
Female	129 (38)	0.55	69 (11.6)	0.39
Total	200 (39.5)		90 (12.2)	

* Results of Chi-square tests by *P* value of <0.05 as significant difference

4. Discussion

During the present study, the results indicated that cancer patients have higher *T. gondii* seroprevalence compared to controls (39.5 % in the cancer patients and 12.2 % in controls) which indicate that cancer patients are at a higher risk for acquiring toxoplasmosis than normal individuals as reported by a previous study (Wang *et al.*, 2017). The seroprevalence observed in this study was close to the *T. gondii* seroprevalence observed in a case–control study of nine-hundred cancer patients and nine-hundred controls in China where the seroprevalence was (35.56 %) and (17.44 %) in cancer patients and the controls, respectively (Cong *et al.*, 2015). Furthermore, the prevalence in the present study (39.5 %) is higher than that observed in two similar studies on cancer patients in Saudi Arabia and Egypt. Among 137 and 150 cancer patients a seroprevalence of 30.6 % and 20 % was observed in Saudi Arabia and Egypt, respectively (Wassef *et al.*, 2016, Imam *et al.*, 2017). Contrary to that, a previous seroprevalence study on cancer patients in Turkey reported a much higher seroprevalence reaching up to (63.0 %) (Yazar *et al.*, 2004).

In the present study, 2.5 % of the cancer patients and 1.1 % of the controls were positive for *Toxoplasma* IgM antibodies which indicates a recent / current infection. On the other hand, 2.0 % of the cancer patients and 1.1 % of the control had both IgG and IgM antibodies which indicate an old and a current infection. In the present study, it is very important to notice that the cancer patients with positive IgM are presented with new *T. gondii* infection which might be acquired from the environment or during multiple blood transfusions. In such compromised patients, the disease might progress directly to active toxoplasmosis, so immediate treatment is required. A recent case report described a disseminated toxoplasmosis after stem cell transplantation in a leukemia toxoplasmosis seronegative patient (Osthoff *et al.*, 2013). The prevalence of toxoplasmosis is observed to be high in general among immunocompromised groups. One study investigated 394 Chinese patients at intensive care units (ICU), and found that 18.78 % of them were positive for anti-*T. gondii* IgG antibodies demonstrating a latent infection (Zhang *et al.*, 2015).

Consistent with many previous studies, the present study observed that the seroprevalence of toxoplasmosis increased with age in both cancer patients and the controls where the highest seropositivity was observed with age >51 (Zhang *et al.*, 2015, Imam *et al.*, 2017). The rise in seroprevalence with age might be a reflection of the increase in the exposure and greater chance to *T. gondii* infection, as the human being gets older (Imam *et al.*, 2017). Also, multiple minor infections might at first produce low antibody levels which are not detectable and which may later reach higher detectable levels as the individual ages (Robert-Gangneux and Darde, 2012). In the current study, there was no statistically significant difference in toxoplasmosis seroprevalence between the recruited males and females in both cancer patients and the controls. However, in many other previous studies, the prevalence of toxoplasmosis was said to be affected by the sex of the individual; some indicated higher seroprevalence in males compared to females, while others showed the opposite indicating that the seroprevalence is

not affected by the sex of the individual (Konishi *et al*., 2000, Imam *et al*., 2017). It is worth mentioning that the seropositivity is not always an accurate evidence of a latent infection in cancer patients since they might receive multiple blood transfusions (from seropositive donors), that could lead to passively transferred antibodies (Imam *et al.*, 2017, Fallahi *et al.*, 2018).

A pervious study in Egypt indicated that toxoplasmosis was higher in patients having solid organ tumors (24 %) than in patients with hematological malignancies (12 %) (P = 0.06) (Wassef *et al*., 2016). The present study showed that the seropositivity was higher in lung, uterine, thyroid and lymph node cancers (50 %) compared to other types of cancer; however, actually no convincing reason can be seen to explain the difference.

In conclusion, the results of the present study showed that cancer patient are at a higher risk for acquiring toxoplasmosis than normal individuals, and these immunocompromised cancer patients should be routinely screened for this parasite in order to put them on early therapy, and prevent severe dissemination. Physicians should start a treatment protocol if anti-*Toxoplasma* IgM antibodies were detected in the cancer patient's serum to avoid any complications of toxoplasmosis.

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A Comparison of the Biological Activities of *Citrus sudachi* Hort. ex Shirai Peels Grown in Japan and Korea

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Abstract

This study evaluates the *Citrus sudachi* Hort. ex Shirai (sudachi) peel grown in Japan and Korea as an antioxidant, an antimicrobial, and *α*-glucosidase inhibitory agent. In this investigation, 80% methanol (MeOH) partitioned into four different fractions—*n*-hexane, ethyl acetate (EtOAc), *n*-butanol, and aqueous, were used as solvents for sudachi. It was found that the *n*-butanol fraction was the highest among the 1,1-diphenyl-2-picryl-hydrazyl (DPPH) radical scavenging assays for both countries. For the reducing power assays, the EtOAc fraction showed the highest reducing power for both countries. The highest phenol and flavonoid content was found in the EtOAc fraction in the samples from both Korea and Japan. For the *α*-glucosidase inhibitory activity, Japanese sudachi demonstrated greater inhibitory activity than Korean sudachi. For the minimum inhibitory concentration (MIC) assay, gram-positive bacteria were more sensitive than gram-negative bacteria, and the EtOAc fraction showed greater inhibitory activity in samples from both countries; however, Korean sudachi exhibited the greatest inhibitory activity. The combined data from all assays indicated that sudachi can be used effectively as a natural antioxidant, antimicrobial, and *α*-glucosidase inhibitory agent.

Keywords: Sudachi peel, Biological activities, Polyphenolics, Different solvents

1. Introduction

Reactive oxygen species (ROS), such as the superoxide anion (O^-), the peroxyl radical (ROO), nitric oxide (NO), and the hydroxyl radical (ROC) are constantly produced endogenously through biological activity (Menković *et al*., 2014). Although antioxidants are also produced in the body (Hwang *et al*., 2013), the over production of ROS results in antioxidative stress, which is expressed as an imbalance of ROS and antioxidant concentrations (Kücükakin *et al*., 2009). These unstable molecules lack one electron in their outer shell and seek to stabilize by stealing an electron from healthy cells around them (Jiménez-Monreal *et al*., 2009). The cells that lose the electron are damaged causing adverse effects for humans, including asthma, diabetes, and cancer (Sindhi *et al*., 2013). These diseases are somehow induced by ROS. Protection against ROS can be accelerated by the intake of antioxidants and the most commonly used synthetic antioxidants are butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA), which are used as food additives, or to slow the deterioration of cosmetics (Sindhi, *et al*., 2013). However, Anbudhasan *et al*. (2014) suggested that the BHT and BHA could have negative health effects. Accordingly, consumer interests shifted to antioxidants from natural foods, which have no reported adverse side effects. Many studies have described the beneficial antioxidants in foods such as cereals, herbs, vegetables, fruits, and medicinal plants (Singh and Kumari, 2015; Djordjevic *et al*., 2011; Jayanthi and Lalitha, 2011; Akdaş and Bakkalbaşı, 2017; Jamuna *et al*., 2010). Ascorbic acid and *α*-tocopherol, commonly known as vitamin C and vitamin E, respectively are two of the most well-known natural antioxidants. In recent years, much more attention has been focused on citrus fruits due to their high concentration of antioxidants and their anticancer properties (Karsheva *et al*., 2013; Entezari *et al*., 2009).

Citrus sudachi Hort. ex Shirai (sudachi) belongs to the Rutaceae family and is originally from Japan. Sudachi is mostly known in the southern parts of Japan. Unlike the orange, its fruit is harvested before it has completely ripened. The taste of sudachi is relatively bitter and gives off a unique aroma (Kim and Kim, 2016). This strong aroma is one of the key characteristics of sudachi, as also is its high levels of vitamin C (Lee *et al*., 2015). Recent reports suggest that the constituents of sudachi reduce the risk of hyperglycemia and lower the serum glucose level in obese individuals (Akaike *et al*., 2014). Sudachitin, a unique component found only in sudachi peel, increases energy expenditure and weight loss by increasing metabolism and stimulating mitochondrial biogenesis (Tsutsumi *et al*., 2014). The ability of sudachi to inhibit bacterial growth has also been investigated (Tomotake *et al*., 2006).

Sudachi is generally consumed as an acidulant in savory foods or juice. In both cases, the fruits are used, and

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the peel is generally discarded as industrial waste by the factories that process them. In Japan, the discarded sudachi peel gives off an unpleasant odor, and this has become a significant problem, both financially and environmentally, for the producers and processors (Tsukayama *et al*., 2010). In contrast, sudachi is much less popular in Korea, and it is consumed in very small amounts. Although some studies describe the antioxidative, antidiabetic, and antimicrobial activity of sudachi, there are no reports that compare sudachi from Japan with sudachi from Korea. Thus, the aim of this study is to investigate the efficacy of sudachi peel as a natural antioxidant by comparing the sudachi from these two countries. Strong antimicrobial and *α*-glucosidase inhibitory activity has also been reported with regard to sudachi peel (Ali *et al*., 2017; Vasu *et al*., 2017), and these inhibitory activities were also evaluated.

2. Materials and Methods

2.1. Chemicals

Potassium ferricyanide, trichloroacetic acid, 1,1-diphenyl-2-picryl-hydrazyl (DPPH), ferric chloride, sodium carbonate, and BHT were purchased from Wako Pure Chemical Industries Ltd. (Japan). Quercetin, gallic acid, arbutin, and 4-Nitrophenyl-β-D-glucopyranoside (*p*NPG) were obtained from Sigma Chemical Co. (USA). Other reagents were obtained from Daejung Chemical and Metal Co., Ltd. (Republic of Korea). All reagents were of analytical grade.

2.2. Plant Material and Extraction

Two samples from Japan and Korea were collected for this study. The sudachi grown in Japan was obtained from the Tokushima prefecture on September 10th, 2014. Another sample was collected from Jeju Island in Korea on September 22nd, 2014. Both samples were peeled, and the peels were dried under ambient temperature conditions. After drying, the samples were crushed by a mixer to make a powder. Each sample was extracted with 900 mL of 80% methanol (MeOH) solution with 64 g of pulverized Japanese sudachi peel (145 g of pulverized Korean sudachi peel) and were put in an ultrasonic bath (Power Sonic 520, Hwashin, Co., Korea) for ninety minutes. This process was continued three times. The solution was then filtered to remove impurities and the extracts were evaporated by a rotary vacuum evaporator (Hei-VAP Precision 280 rpm, Heldolph, Germany). The concentrate was set aside, and the remnants were dissolved by water and partitioned with *n*-hexane, ethyl acetate (EtOAc), *n*-butanol, and distilled water (aqueous) sequentially. The samples were stored in a refrigerator at -20ºC until further analysis.

2.3. Analysis of 1,1-diphenyl-2-picryl-hydrazyl (DPPH) Radical Scavenging Activity

A DPPH assay was performed using Hyun *et al*.'s method (2014), with slight modification. Briefly, each diluted sample (100 µL) was placed in a test tube. The MeOH solution was added to each sample test tube to adjust the total volume to 4 mL. One hundred µL of DPPH (0.15 mM) was added to each mixed solution and incubated in a dark room for thirty minutes. After thirty minutes, the absorbance was measured with a UV-Spectrophotometer at 517 nm (UV-1800, Shimadzu Co., Japan) against MeOH as the blank. Lower UV-Spectrophotometer readings indicate greater DPPH radical scavenging activity. The scavenging activity was calculated according to the following equation:

$$\% \text{ inhibition} = (1 - A_S / A_C) \times 100$$

(where A_C is the absorbance of the negative control [without sample extract], and A_S is the actual absorbance value of each sample and the positive control [BHT, *α*-tocopherol, and ascorbic acid]). Also, BHT, ascorbic acid, and *α*-tocopherol were used as positive controls.

2.4. Measurement of Reducing Power Assay

The reducing power assay was performed using the method described by Nakamura *et al*. (2017). The samples (10, 20, and 30 µL) were each dispensed to a test tube, and the total volume of each sample was adjusted to 100 µL with distilled water. Then, 500 µL of 1% potassium ferricyanide and 0.2 M of sodium phosphate buffer (pH = 6.6) were added to the mixed solution. The samples were allowed to react at 50°C for twenty minutes. The resulting solution was spiked with 2.5 mL of 10% trichloroacetic acid to stop the reaction. The supernatant (500 µL) was transferred into the test tube and mixed with 500 µL of distilled water. Finally, 0.1% ferric chloride was added to the mixed solution. The absorbance was measured at 700 nm. Higher absorbance rates indicate a greater reducing power. Alpha-tocopherol and BHT were used as standards.

2.5. Determination of Total Phenol and Flavonoid Contents

The total phenol content was measured using the Folin–Ciocalteu method employed by Nakamura *et al*. (2016). One hundred µL of each sample or a standard solution of gallic acid to draw the calibration curve was mixed thoroughly with 50 µL of Folin–Ciocalteu reagent for five minutes. Then, 300 mL of 20% sodium carbonate was added to the solution, and the mixture was allowed to react for twenty minutes at ambient temperature. Prior to measuring the absorbance rate at 725 nm by UV-Spectrophotometer, 1 mL of distilled water was added. The data were expressed as milligrams of gallic acid equivalent (GAE) per gram of the sudachi extract, based on the calibration curve of gallic acid (mg GAE/g of sudachi).

The total flavonoid content was estimated using the aluminum chloride colorimetry method described by Nakamura *et al*. (2016). In brief, to draw the calibration curve, a 6-fold serial diluted solution with quercetin was prepared (3.125, 6.25, 12.5, 25, 50, 100 mg/L). Simultaneously, the 20 µL of samples were diluted with 180 µL of 80% EtOH. Each 200 µL diluted sample and standard solution was transferred to a test tube and mixed with 100 µL of 10% aluminum nitrate. Then, 100 µL of 1 M potassium acetate and 4.6 mL of 80% ethanol were added to the solutions. After keeping the solution at room temperature for forty minutes, the maximum absorbance of each mixture was measured by UV-Spectrophotometer at 417 nm, and a comparison with the standard curve obtained from quercetin was done. The total flavonoid content was described as milligrams of quercetin equivalent (QE) per gram of sudachi extract, based on the calibration curve of quercetin (mg QE/g of sudachi).

2.6. Assay for α-Glucosidase Inhibitory Activity

The *α*-glucosidase inhibitory activity assay employed the method used by Yang *et al*. (2011) with a minor modification. Fifty µL of the sample solution and sodium phosphate buffer (0.2 M, pH = 6.8) were added to each

microtube. Then, prior to incubating for fifteen minutes at 37°C, 50 µL of *α*-glucosidase (0.5 U/mL) was mixed with each sample solution. The substrate *p*NPG (100 µL, 0.3 mM) was allowed to react with the reaction mixture for ten minutes at 37°C. The reaction was forced to terminate by adding 750 µL of Na_2CO_3 (0.1 M). Finally, the absorbance was measured at an absorbance intensity of 405 nm. Alpha-glucosidase inhibitory activity was calculated using the equation:

$$\% \text{ of inhibition} = (1 - (As / Ab) / Ac) \times 100$$

The control was prepared by including all reagents without a sample solution, whereas the blank was all reagents without *p*NPG. As a positive control, acarbose was used. The absorbance of the test samples was presented with the absorbance of the blank solution (Ab) and the absorbance of the control without samples (Ac).

2.7. Determination of Minimum Inhibitory Concentration

To determine the minimum inhibitory concentration (MIC) of each extract and each fraction, we performed a serial two-fold dilution method, adapted from Jeong *et al.* (2010), using 96 well microtiter plates. First, 180 µL of an appropriate medium with bacteria and 20 µl of sample were added to the first row. Then, 100 µL of the medium was spiked from the second row to the last row. Finally, the mixed medium was diluted sequentially. The inhibition activity was evaluated for tardiness with the naked eye after twenty hours. In the current study, six different kinds of bacteria were used—3 gram-positive bacteria (*Bacillus subtilis* subsp. *spizizenii*, *Staphylococcus epidermidis*, and *Micrococcus luteus*) and 3 gram-negative bacteria (*Salmonella enterica* subsp. *enterica*, *Escherichia coli*, and *Klebsiella pneumonia*). The *Salmonella enterica* subsp. *enterica* samples were incubated at 30°C with nutrient agar, and the other bacteria samples were incubated at 37°C in PP-Medium. All the strains of bacteria used in this study were distributed by the Korean Agricultural Culture Collection (KACC) in Korea.

2.8. Phenolic Compound Evaluation by HPLC

HPLC analysis was performed with a Shimadzu (LC-10ADvp) Liquid Chromatography System using an SPD-10A UV-Vis detector for the extracts. The column (Luna 5µ C18 [2] 100A 205 x 460 mm) was used at 40°C. The elution solvents used were (A) acetonitrile and 0.5% acetic acid and (B) water and 0.5% acetic acid. The solvent gradient was carried out as follows: 0 min, 20–80; 14 min, 20–80; 19 min, 40–60; 33 min, 40–60; 38 min, 70–30; 47 min, 70–30; 50 min, 20–80; and 60 min, 20–80, and the injection volume was 10 µL. The phenolic compounds were identified by the retention time and UV spectra of a standard measured from the peak area at 280 nm.

2.9. Correlation and Data Analysis

All determinations were expressed as the mean ± standard deviation of an average triplicate analysis. The difference between the treatment and control groups were analyzed using ANOVA with Duncan's multiple range test. Significance was expressed by P values, and ANOVA values were considered to be statistically significant when the P value was less than 0.05. Data analysis was performed using the SPSS program (Statistical Package for Social Science, Ver. 20.0 [SAS Institute Inc., Cary, NC, USA]).

3. Results and Discussion

3.1. 1,1-diphenyl-2-picryl-hydrazyl (DPPH) Radical Scavenging Activity

Table 1 shows that the Japanese sudachi exhibits greater DPPH radical scavenging activity than the Korean sudachi. The *n*-butanol fraction from both countries (45.3±2.3 µg/mL for the Korean sudachi and 32.0±2.01 µg/mL for the Japanese sudachi) demonstrated the greatest ability to reduce DPPH radicals, whereas significant DPPH radical scavenging activity was not observed in the *n*-hexane fractions or the aqueous fractions (with the exception of the aqueous fraction from Japan). In addition, only the EtOAc and *n*-butanol fractions in Japanese sudachi were superior to BHT, but they did not show greater antioxidative activity than ascorbic acid and *α*-tocopherol. Kim (2014) performed the DPPH scavenging activity assay to determine the antioxidant ability of *Maesa japonica* (Thunb.) leaves and twigs, and found that the *n*-butanol fraction exhibited the greatest antioxidant activity, while the EtOAc fraction showed a slightly lower value than the *n*-butanol fraction for leaves. His results exhibited a similar trend to the results of the present study. Comparing the Korean and Japanese sudachi, the Japanese sudachi exhibited a greater radical scavenging activity than the Korean sudachi for each of the extracts and fractions. Furthermore, the RC_{50} value of the aqueous fraction in Korean sudachi was much higher than 100 µg/mL. Conversely, the Japanese sudachi exhibited a value less than 100 µg/mL (72.7±13.5 µg/mL). This might explain why the Japanese sudachi has slightly more DPPH radical scavenging activity than the Korean sudachi. Also, it is widely thought that the antioxidant activity is closely related to the total phenolic content (Orhan and Üstün, 2011). According to the current data, the fractions and extracts that contained greater quantities of phenolic compounds also showed a greater radical scavenging ability, but this correlation was not always consistent. In Tables 1 and 2, the EtOAc fractions exhibited the highest phenolic content. In contrast, the *n*-butanol fraction demonstrated the highest DPPH radical scavenging activity, followed by the EtOAc fraction. This small discrepancy might be attributed to the different response to the Folin–Ciocalteu reagent by each of the phenolic compounds (Yu *et al.*, 2002). It may be that the total phenol content assays did not fully reflect the actual total phenolic content in the samples, excluding certain antioxidants such as vitamin C or vitamin E, for example (Saha and Paul, 2014).

Table 1. 1,1-diphenyl-2-picryl-hydrazyl (DPPH) radical scavenging activity in the extract and fractions from *Citrus sudachi* Hort. ex Shirai.

Extract and fractions	RC_{50} (µg/mL)[z]	
	Korea	Japan
80% MeOH extract	60.0±0.9	46.1±0.4
n-Hexane fraction	> 100	> 100
EtOAc fraction	49.3±3.3	37.5±1.4
n-Butanol fraction	45.3±2.3	32.0±2.01
Aqueous fraction	> 100	72.7±13.5
Ascorbic acid	0.6±0.01	
a-Tocopherol	1.2±0.02	
BHT	37.6±0.7	

[z] The amount of RC_{50} required for a 50% reduction of DPPH after 30 min; each value is the mean±standard deviation of triplicate experiments.

3.2. Reducing Ability Assay

The capacity of sudachi to reduce Fe^{3+} to Fe^{2+} is shown in Figure 1. As is evident, among the extracts and fractions, the greatest reducing power was observed for the EtOAc fraction, followed by the *n*-butanol fraction, the 80% MeOH extract, the aqueous fraction, and the *n*-hexane fraction. There is agreement between the samples from both countries, and, according to these data, there is no significant difference between Korean and Japanese sudachi, except for the EtOAc fraction; in this case, Japanese sudachi exhibited a greater reducing ability than Korean sudachi. Although the EtOAc fractions from both countries exhibited the highest reducing ability relative to the other fractions, their values were not higher than the reference compounds (*α*-tocopherol and BHT). However, the 30 µg/mL concentration of the EtOAc fraction for the Japanese sudachi exhibited a similar reducing ability to that of the 20 µg/mL concentration of *α*-tocopherol and BHT. Venkatachalam and Muthukrishnan (2012) indicated that inherent reductive components in the sample serve as useful indicators of potential antioxidative activity. In the EtOAc fraction, there might be increased reduction due to vitamin C, for example, which has a ketone and enediol structure.

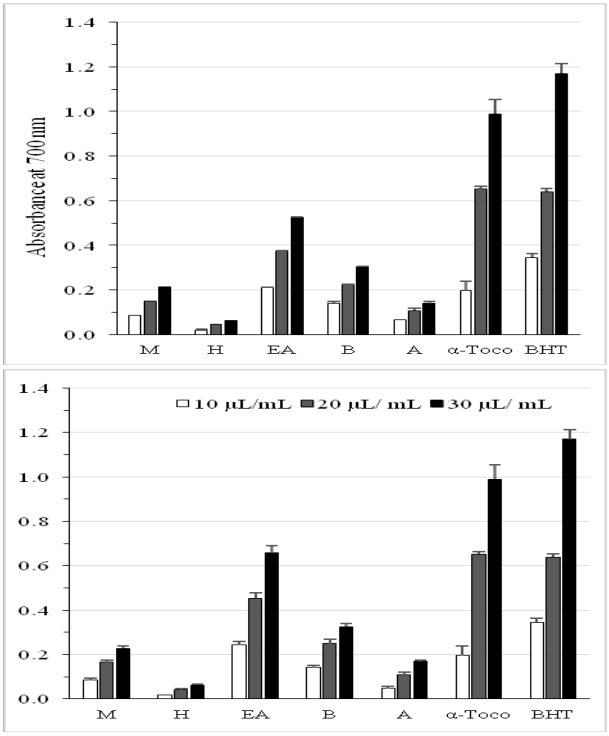

Figure 1. The reducing power of *Citrus sudachi* Hort. ex Shirai in Korea (up) and Japan (down). M: 80% MeOH; H: *n*-hexane; EA: EtOAc; B: *n*-butanol; A: aqueous; *α*-Toco: *α*- tocopherol.

3.3. Total Phenol and Flavonoid Content

According to Table 2, the highest phenol concentrations were in the EtOAc fractions (2,420.0±17.5 mg GAE/g for the Korean sudachi and 2,041.6±96.0 mg GAE/g for the Japanese sudachi). The *n*-hexane fraction exhibited the lowest phenol content, with values of 204.9±5.3 mg GAE/g for the Korean sudachi and 192.6±12.8 mg GAE/g for the Japanese sudachi. Comparing Korean and Japanese sudachi, Korean sudachi showed a higher total phenol content in 80% MeOH extract (1,157.1±17.5 mg GAE/g), the EtOAc fraction (2,420.0±17.5 mg GAE/g), and the *n*-butanol fraction (2,011.0±105.1 mg GAE/g), whereas the Japanese sudachi showed a higher total phenol content in the aqueous fraction (707.1±32.0 mg GAE/g). For the flavonoid content in the Korean sudachi, the values ranged from 12.4±0.0 mg QE/g for the aqueous fraction to 445.9±12.5 mg QE/g for the EtOAc fraction. For the Japanese sudachi, the flavonoid content ranged from 63.2±3.1 mg QE/g for the aqueous fraction to 496.8±15.6 mg QE/g for the EtOAc fraction. Both Korean and Japanese sudachi demonstrated the highest total flavonoid concentration in the EtOAc fraction and the lowest in the aqueous fraction. Interestingly, although the *n*-hexane fraction exhibited the lowest total phenol content, it showed the second highest total flavonoid content. Overall, Japanese sudachi exhibited a slightly higher flavonoid content. As illustrated in Table 2, these trends are in agreement with the report from Ao *et al*. (2008). In addition, these data (2,420.0±17.5 mg GAE/g for the Korean sudachi and 2,041.6±96.0 mg GAE/g for the Japanese sudachi in the EtOAc fraction; and 204.9±5.3 mg GAE/g for the Korean sudachi and 192.9±12.8 mg GAE/g for the Japanese sudachi in the *n*-hexane fraction) were higher than those reported by Jan *et al*. (2013) (31.5±2.4 mg GAE/g for the EtOAc fraction and 16.7±1.3 mg GAE/g for the *n*-hexane fraction). The higher phenolic content might be attributed to their hydroxyl groups, which are directly related to the antioxidant activity. Hydroxyl groups have the important role of eliminating ROS. However, these special structures are capable of chelating with metal ions, which are responsible for producing ROS, thus revealing greater antioxidant activity (Heijne *et al*., 2001). The fractions showing a higher total phenol content in Table 2 might contain high levels of hydroxyl groups.

For the total flavonoid content, Ghasemi *et al*. (2009) investigated the peel and tissue of thirteen citrus fruits, including lemon, grapefruit, and oranges, and pointed out that the peel had a higher flavonoid content. However, the flavonoid content obtained in the present investigation was higher than their result, except for the aqueous fraction of Korean sudachi. In addition, Khatiwora *et al*. (2010) determined the total flavonoid content of *Ipomoea carnea* leaves, stems, and flowers. The results indicated that all parts had a relatively high flavonoid content. The flowers had the highest flavonoid content (422 mg QE/g); these values are similar to values from the present study for the EtOAc fractions in Korean and Japanese sudachi.

Table 2. Total phenol and flavonoid content of *Citrus sudachi* Hort. ex Shirai extract and its fractions.

Extract and fraction	TPC[z] (mg GAE/g)		TFC[zz] (mg QE/g)	
	Korea	Japan	Korea	Japan
80% MeOH extract	1,157.1±17.5	1,091.6±103.7	123.0±6.3	125.2±3.1
n-Hexane fraction	204.9±5.3	192.9±12.8	169.4±3.1	213.7±3.1
EtOAc fraction	2,420.0±17.5	2,041.6±96.0	445.9±12.5	496.8±15.6
n-Butanol fraction	2,011.0±105.1	2,002.0±121.6	138.5±9.4	169.4±9.4
Aqueous fraction	464.3±10.5	707.1±32.0	12.4±0.0	63.2±3.1

[z] TPC: Total phenolic content. Total phenolic content analyzed as gallic acid equivalent (GAE) mg/g of extract; values are the average of triplicates.

[zz] TFC: Total flavonoid content. Total flavonoid content analyzed as quercetin equivalent (QE) mg/g of extract; values are the average of triplicates.

3.4. α-Glucosidase Inhibitory Activity Assay

In this study, to evaluate the degree to which the samples exhibited the release of *p*-nitrophenol from *p*NPG, the IC_{50} value was calculated. As shown in Table 3, the inhibitory range is from 356.6±2.5 µg/mL to 41.9±0.5 µg/mL for Korean sudachi and 346.8±8.8 µg/mL to 40.0±0.7 µg/mL for Japanese sudachi. The *n*-butanol fraction exhibited the highest *α*-glucosidase inhibitory activity (41.9±0.5 µg/mL for Korean sudachi and 40.0±0.7 µg/mL for Japanese sudachi), while the 80% MeOH extract showed the lowest inhibitory activity (356.6±2.5 µg/mL for the Korean sudachi and 346.8±8.8 µg/mL for the Japanese sudachi). The *n*-hexane and aqueous fractions from both countries did not show any significant activity under the set environment. Japanese sudachi demonstrated greater inhibition than Korean sudachi. The *n*-butanol and EtOAc fractions of Korean and Japanese sudachi showed greater inhibition than that of acarbose (90.8±1.8 µg/mL), but the other extracts and fractions showed lower values than that of the positive reference. Jeong *et al*. (2013) performed *α*-glucosidase activity assays with *Rehmannia glutinosa* tuberous roots, discovering that acetone and EtOAc fractions showed poor *α*-glucosidase activity. These same fractions possessed the highest and second highest phenol (flavonoid) content. Their study suggested that phenol and flavonoid probably do not interact with the activity of *α*-glucosidase, and may contain non-polyphenolic active compounds such as polysaccharide (Chen *et al*., 2009). However, the present study exhibited results that were more similar to those described by Wongsa *et al*. (2012), as the EtOAc or *n*-butanol fraction demonstrated greater phenol and flavonoid content and *α*-glucosidase activity. Through this study, it is predicted that a higher phenol and flavonoid content leads to a greater potential *α*-glucosidase activity.

Table 3. The IC_{50} values of *α*-glucosidase inhibitory activity assay of the extracts and fractions**.**

Extract and fractions	IC_{50} (µg/mL)[z]	
	Korea	Japan
80% MeOH extract	356.6±2.5	346.8±8.8
n-Hexane fraction	> 500.0	> 500.0
EtOAc fraction	55.1±1.3	52.5±1.5
n-Butanol fraction	41.9±0.5	40.0±0.7
Aqueous fraction	> 500.0	> 500.0
Acarbose	90.8±1.8	

[z] IC_{50}: The concentration required to inhibit 50% *α*-glucosidase activity under the study condition.

3.5. Minimum Inhibitory Concentration (MIC) Determination

The MIC of the extracts and fractions against the tested strains are presented in Table 4. As shown, the 80% MeOH extract, the *n*-butanol fraction, and the aqueous fraction exhibited low inhibitory activity, whereas the *n*-hexane and EtOAc fractions showed some inhibitory activity. Inhibitory activity was found to be particularly high with the *n*-hexane and EtOAc fractions against *Staphylococcus epidermidis*. Also, the gram-positive bacteria exhibited more sensitivity than the gram-negative bacteria. Overall, Korean sudachi exhibited greater inhibition activity compared to the Japanese sudachi, and the inhibitory effect of Korean sudachi for the EtOAc fraction against *Bacillus subtilis* subsp. *spizizenii* and *Staphylococcus epidermidis* were twice that of Japanese sudachi. According to Blanco *et al*. (2005), antimicrobial activity mostly relies on phenol content, such as epigallocatechin gallate; however, the current study indicates otherwise. Table 2 shows that the EtOAc fraction had the highest total phenol content followed by the *n*-butanol fraction; however, the *n*-butanol fraction demonstrated a low antimicrobial effect against all tested bacterial strains, while the *n*-hexane fraction inhibited *Staphylococcus epidermidis* at a concentration of 500 µg/mL. Perhaps other compounds that could not be measured in the total phenol and flavonoid assay may also work as antimicrobial agents (Hyun *et al*., 2014). Also, it is believed that gram-positive bacteria are more susceptible to antimicrobial effects due to differences in their cellular mechanisms compared to gram-negative bacteria (Malanovic and Lohner, 2016). Though Çördük *et al*. (2017) previously reported that *Digitalis trojana* Ivanina plant extracts effectively inhibited the growth of gram-negative bacteria, sudachi may contain compounds that interfere with the antimicrobial defense of gram-positive bacteria. Singh *et al*. (2016) also reported the more inhibition of gram-negative bacteria from the *Sapindus mukorossi* plant extracts. Furthermore, Tomotake *et al*. (2006) reported that sudachi favorably constrained eight species of *Vibrio* strains multiplication when using disk diffusion methods (a clear zone of >5 mm in diameter for *V. alginolyticus* 6624 and *V. anguillarum* NCMB 829). These findings support the conclusion that most effective antimicrobial compounds are in the EtOAc fraction.

Table 4. The minimum inhibitory concentration (MIC) of each extract and fraction against selected strains of bacteria.

	Extracts and fractions	MIC (μg/mL)					
		B.S (+)	S.Ep (+)	M.L (+)	S.En (-)	E.C (-)	K.P (-)
Korea	80% MeOH extract	-	-	-	-	-	-
	n-hexane fraction	-	500.0	-	-	-	-
	EtOAc fraction	62.5	125.0	500.0	-	-	-
	n-butanol fraction	-	-	-	-	-	-
	Aqueous fraction	-	-	-	-	-	-
Japan	80% MeOH extract	-	-	-	-	-	-
	n-hexane fraction	-	500.0	-	-	-	-
	EtOAc fraction	125.0	250.0	500.0	-	-	-
	n-butanol fraction	-	-	-	-	-	-
	Aqueous fraction	-	-	-	-	-	-

B.S: *Bacillus subtilis* subsp. *spizizenii* KACC 14741; S.Ep: *Staphylococcus epidermidis* KACC 14822; M.L: *Micrococcus luteus* KACC 14819; S.En: *Salmonella enterica* subsp. *enterica* KACC 10769; E.C: *Escherichia coli* KACC 14818; K.P: *Klebsiella pneumoniae* KACC 14816; - : > 1,000 μg/mL.

3.6. Analysis of Phenolic Compounds by HPLC

Four phenolic compounds were identified from the retention time and UV spectra of standard as summarized in Table 5. The four phenolic compounds identified by HPLC were rutin, naringin, hesperidin, and hesperetin. The retention time for each of the flavonoids was: rutin, 7.73 minutes for the Korean sudachi and 7.52 minutes for the Japanese sudachi; naringin, 13.57 minutes for both countries; hesperidin, 16.78 minutes for both countries; and hesperetin, 27.95 minutes for the Korean sudachi and 27.98 minutes for the Japanese sudachi. There was no significant difference in the naringin content between the Korean and Japanese samples. Hesperidin showed the highest content of all four flavonoids identified. Korean sudachi contained more hesperidin and hesperetin (33.6±3.2 μg/mg and 3.5±0.2 μg/mg, respectively), whereas Japanese sudachi contained more rutin (14.6±0.4 μg/mg). Nakagawa *et al*. (2006) isolated twenty-seven known compounds from the peel of sudachi—including naringin, hesperidin, hesperetin, and five new compounds—and analyzed their structural composition. In the present study, however, rutin was detected. It can be assumed that this is related to a difference in fruit quality, variety, harvest season, or the sample preparation method (Kumazawa *et al*., 2007). Nogata *et al*. (2006) isolated compounds from forty-two citrus fruits, and found that the peel of sudachi contained greater amounts of rutin (121 mg/100g fresh weight), naringin (70.7 mg/100g fresh weight), and hesperidin (38.9 mg/100g fresh weight). Nonetheless, the current study shows that hesperidin had the highest amount (33.6±3.2 μg/mg DW for the Korean sudachi and 25.2±0.2 μg/mg DW for the Japanese sudachi), followed by naringin (15.1±0.9 μg/mg DW for Korean sudachi and 15.1±0.3 μg/mg DW for the Japanese sudachi), rutin (10.6±0.6 μg/mg DW for the Korean sudachi and 14.57±0.42 μg/mg DW for Japanese sudachi), and hesperetin (3.5±0.2 μg/mg DW for the Korean sudachi and 1.8±0.1 μg/mg DW for the Japanese sudachi).

Rutin is known as a strong antioxidant that attenuates senescence. According to Yang *et al.* (2008), rutin demonstrated greater DPPH radical scavenging activity and reductive capability than vitamin C and BHT. In their study, at a concentration of 50 μg/mL, rutin showed a slightly lower value than vitamin C (92.8% and 90.4%, respectively) but a much higher value than BHT (58.8%). These data clearly demonstrate that rutin has significant properties as a radical inhibitor or scavenger. Through the HPLC analysis in this study, rutin was found to be 10.6±0.6 μg/mg DW for the Korean sudachi and 14.6±0.4 μg/mg DW for the Japanese sudachi, revealing that Japanese sudachi contains 28% more rutin than Korean sudachi. This fact may be one of the reasons that Japanese sudachi exhibited greater DPPH radical scavenging activity and reducing ability in the current investigation. Furthermore, hesperetin, which has a low concentration in citrus fruits, may play an important role as a potent antimicrobial agent. Moon *et al*. (2013) performed a disk diffusion assay with three strains of *H. pylori* and showed more than 20 mm inhibition at concentrations ranging from 5 to 20 mM. This study showed that Korean sudachi demonstrated marginally greater inhibitory activity against *Bacillus subtilis* subsp. *spizizenii* and *Staphylococcus epidermidis*. Hesperetin may be partially responsible for the antimicrobial activity in the present study.

Table 5. Analysis of flavonoid for *Citrus sudachi* Hort. ex Shirai by HPLC.

	Compounds	Retention time (min)	Content μg/mg DW
Korea	Rutin	7.73	10.6±0.6
	Naringin	13.57	15.1±0.9
	Hesperidin	16.78	33.6±3.2
	Hesperetin	27.95	3.5±0.2
	Total		62.7
Japan	Rutin	7.52	14.6±0.4
	Naringin	13.57	15.1±0.3
	Hesperidin	16.78	25.2±0.2
	Hesperetin	27.98	1.3±0.1
	Total		56.7

3.7. Correlation among Phenolic Contents, Antioxidative, and α-Glucosidase Inhibitory Activity

To understand the relationships between the phenolic compound content and DPPH radical scavenging activity, reducing power, and α-glucosidase inhibitory activity, correlation analyses were performed. Table 6 shows the intercorrelation between the phenolic compound content and each assay. As displayed in Table 6, the total phenolic compound content is strongly correlated with DPPH radical scavenging activity (-0.762, $P < 0.001$), reducing power (0.879, $P < 0.001$), and α-glucosidase inhibitory activity (-0.97, $P < 0.001$). These negative correlations indicate that the extracts and fractions of sudachi with a

greater total phenolic compound content exhibit greater antioxidative and α-glucosidase inhibitory activity; conversely, the extracts and fractions with the lowest phenolic compound content showed the lowest reducing ability. As for the total phenolic compound content, the total flavonoid content was also highly correlated with the reducing power (0.806, $P < 0.001$) and the α-glucosidase inhibitory activity (-0.613, $P < 0.010$). However, the total flavonoid content and the DPPH radical scavenging activity (-0.15, $P > 0.050$) demonstrated a poor correlation. These results showed that the total content of phenol and flavonoid is strongly correlated with the inhibition of α-glucosidase, and in particular the total phenol content influences the antioxidative activity, as shown by Beretta *et al*. (2017) and Ngamdee *et al*. (2016). These findings, which correlated linearly between each parameter, are similar to the results obtained from pressurized methanolic extracts of oenological wood (Alanon *et al*., 2015), indicating that the linear coefficient may be considered as a useful indicator to evaluate the antioxidative activity.

Table 6. The correlation between antioxidative, α-glucosidase inhibitory assay, and total phenol and flavonoid**.**

	DPPH	RP[z)]	TPC	TFC	IC_{50}[zz)]
DPPH	1.000	-0.659**[zzz)]	-0.762***[zzzz)]	-0.150	0.646**
RP		1.000	0.879***	0.806***	-0.850***
TPC			1.000	0.593**	-0.970***
TFC				1.000	-0.613**
IC_{50}					1.000

[z)] RP: The value of the reducing power assay.

[zz)] IC_{50}:The value of α-glucosidase inhibitory assay.

[zzz)] **: Significance $P < 0.010$ compared to control.

[zzzz)] ***: Significance $P < 0.001$ compared to control.

4. Conclusions

This study presented a promising prediction regarding the effective use of sudachi peel in terms of its antioxidative effects, antimicrobial properties, and the inhibition of α-glucosidase. To compare the sudachi from Korea with that of Japan, the researchers investigated various *in vitro* assays—the DPPH radical scavenging assay, reducing power assay, total phenol and flavonoid assays, α-glucosidase inhibitory activity assay, and MIC assay. The findings in this study suggest that the EtOAc and *n*-butanol fractions demonstrated higher activities than the MeOH extract and other fractions. Also, the Japanese sudachi exhibited greater antioxidative and α-glucosidase inhibitory activities than Korean sudachi, while Korean sudachi demonstrated greater microbial inhibition. These activities mostly rely on phenol compounds. This is the first report that compares sudachi from Korea to that of Japan. The results of this study provide data about the potentially beneficial compounds contained in sudachi and could be used to help develop effective methods for utilizing the abundant sudachi peel waste coming from many processing facilities.

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Immunological Influences of Experimental Aflatoxicosis in Broilers and the Protective Role of Mannan Oligosaccharides

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Abstract

The present study is carried out to highlight the toxic effects of Aflatoxin B1 on humoral and cell-mediated immune responses of broilers, and to evaluate the efficacy of Mannan oligosaccharides (MOS) to ameliorate these toxic effects. In this study, experimental diets were designed, and the broiler chicks were divided into four groups. The first group was fed a normal diet. The second group was fed normal diet with MOS. The third group was fed aflatoxin B1 at the dose of (250 parts per billion"ppb"), while the last group was fed diet containing the aflatoxin in addition to MOS. The birds were checked for humoral and cell-mediated assays. The results revealed that there was a significant decreasing effect of 250 ppb AFB1 on both the antibody titer against the Newcastle disease virus (NDV) and lymphocyte blastogenesis and transformation. The investigation of differential leukocytic counts revealed lymphocytopenia and heterophilia. Dietary supplementation of a 1g MOS/Kg diet during aflatoxicosis could ameliorate some of the toxic effects of AFB1 in broilers, and improve the immune response. These outcomes confirm the immunosuppressive impact of aflatoxin and the role of mannan oligosaccharides to overcome its adverse effects.

Keywords: Aflatoxicosis, Lymphocyte, HPLC, TLC, Broilers, Mannan

1. Introduction

Mycotoxins are chemically stable, secondary toxic metabolites which are produced by certain fungi. They constitute serious and ever-present environmental health hazards as may maintain their toxic effects over an extended period of time. In addition to their economic impact as they cause several hundred million dollar losses annually in the poultry industries, mycotoxins residues in poultry meat and eggs represent a concern for human health since they are known to have strong hepatotoxic and carcinogenic effects (Zain, 2011).

Aflatoxins are one of the most widespread mycotoxins in poultry and human food that contribute to significant health disorders and decrease in the production performance. Moreover, they have been proven to be carcinogenic and/or toxic to humans and animals as well (Mazzoni *et al*., 2011)**.**

The name ''aflatoxin" is derived from the first letter of the word *Aspergillus* and the first three letters of *flavus*, because it is mostly produced from two species, *Aspergillus flavus* and *Aspergillus parasiticus*, which thrive under hot and humid conditions in the fields and stores where contamination is usually a consequence of interactions among the causative fungi, the host animal and the environment. The response to mycotoxin contamination also depends on a number of factors including the level of exposure, environmental conditions, the type and susceptibility of the invader species (Richard, 2007).

In poultry, aflatoxicosis causes listlessness, anorexia with a low growth rate; poor feed utilization, decreased egg production and increased mortality. In addition to anemia, reduction in the immune function, hepato-toxicosis, hemorrhage, teratogenesis, carcinogenesis and mutagenesis (Oguz, 2012). Among the known aflatoxins, AFB1 is the most commonly encountered and the most potent among the naturally occurring carcinogens, and is classified as a group I carcinogen by the International Agency for Research on Cancer (Yunus *et al*., 2011).

In general, mycotoxins cannot be immediately detected by visual examination; nonetheless, they are revealed when poultry are, for instance, faced with infectious agents, and this might be contributing to their immunosuppression effect (Girish and Smith, 2008).

Accordingly, due to their health problems in poultry flocks and the potential economic losses, poultry producers tried several strategies in order to avoid mycotoxicosis, aiming to protect their flocks against the adverse action of the toxins. In this context, mannan oligosaccharides (MOS) has been proposed as a mycotoxin binder for preventing the adverse effects of mycotoxins in poultry feeds (Pazhanivel and Balachandran 2014)**.**

Mannan oligosaccharides (MOS) is polysaccharide-protein complex derived from the cell wall of

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Saccharomyces cerevisiae. The MOS product is reported to have at least three probable modes of action by which the broiler performance is improved by the adsorption of pathogenic bacteria containing type 1 fimbriae with mannose-sensitive lectins, and by improving the intestinal function or "gut health" in addition to the immune modulation and simulating gut associated and systemic immunity by acting as a non-pathogenic microbial antigen, giving an adjuvant-like effect (Spring *et al*., 2000).

Considering the above-mentioned facts, the present study was planned with the aim of studying the impact of aflatoxin (AFB1) on avian immunity and to assess the efficacy of MOS as a mycotoxin binder in counteracting the adverse effects of aflatoxicosis in broilers.

2. Materials and Methods

2.1. Extraction of aflatoxin from Aspergillus flavus (Bauer et al., 1983)

Aspergillus flavus NRRL-500 purchased from the Microbiological Resources Centre (Cairo MIRCEN) was grown on potato dextrose agar slants for approximately fourteen days at room temperature (20-22 ^{o}C) until well sporulated. Spores were harvested, adjusted to approximately 5 X 10^{6} spores/ mL. Spore suspension was cultured and incubated at room temperature (20-22 ^{o}C) in dark conditions for twenty days.

At the end of the incubation period, mycelia of cultures were carefully overlaid with 25 mL chloroform and kept for twenty-four hours in a dark room. Chloroform layers were combined and concentrated in rotary flash evaporator.

2.2. Chromatographic Estimation of Extracted Aflatoxin:

2.2.1. Qualitative Determination of Aflatoxin by Thin Layer Chromatography (TLC) (Gimeno, 1979)

Activated coated silica gel chromatographic plates were spotted parallelly from the chloroform extract together with the standard aflatoxin B1, and were left to dry in the air. The prepared TLC plates were transferred into the developing tank containing the developing solvent system [toluene- ethyl acetate – 90 % formic acid (60:30:10)]. The plates were inspected under UV light (365 nm), and the rate of flow (RF) values, colors, and intensities of the unknown spots were compared with those of the standard ones.

2.2.2. Quantitative Determination of Aflatoxins by High Performance Liquid Chromatography with Postcolumn Fluorescence Derivatization (HPLC-FLD)

Aflatoxin B1, B2, G1 and G2 standards, and HPLC grade solvents were purchased from Sigma, Chemical Co (St. Louis, MO, U.S.A.).

Derivatization of aflatoxins was performed as described in (Manetta *et al*., 2005). The stock solution of aflatoxin standards was dissolved in acetonitrile: methanol (9:1) (AOAC, 2000). HPLC analysis was performed using Agilent Technologies 1100 series liquid chromatograph equipped with an auto sampler and fluorescence detector. The analytical column was Eclipse XDB-C18 (150 X 4.6 µm; 5 µm) with a C18 guard column (Agilent, USA). The mobile phase consists of Acetonitrile / Water / Methanol (1:6:3), then detected at 360 nm fluorescence wave length for excision and 440 nm for emission. The peaks were identified by congruent retention times and were compared with those of the standards.

2.3. Experimental Diet

Known amounts of AFB1 was added and thoroughly mixed with the basal ration (tested negative for the presence of any possible residual mycotoxins) in proportion so as to arrive at the respective dose levels. The amount of crude AFB1 was added at a dose of 250 ppb/kg (parts per billion). Samples were taken from the mixed diet and further quantified for the concentration of the mycotoxin by HPLC to ensure proper mixing.

The experiment was carried on 121-day-old Cobb broiler chicks obtained from Misr Arab Poultry Company. The chicks were acclimatized for a period of one week before the commencement of the trial and were divided into four equal groups, n=30 each, the experimental design was as follows:-

Group 1 (control negative) healthy chicks were given normal ration from one day of age till the end of the experiment.

Group 2 (control positive) healthy chicks were given normal ration containing 1g MOS/kg feed from the second week of age till the end of the experiment.

Group 3 was given a ration containing 250 ppb AFB1/kg feed from the second week of age till the end of the experiment.

Group 4 was given a ration containing 250 ppb AFB1 plus 1g MOS/ kg feed from the second week of age till the end of the experiment.

Immunization: All chicks were vaccinated against Newcastle disease on day seven (Anil *et al*., 2003).

2.4. Assessment of Immunological Parameters

2.4.1. Differential Leukocyte Count

Moderately thin blood films were fixed with methyl alcohol, stained with diluted Giemsa stain for thirty minutes and examined microscopically. The percentage values of different leukocytes were calculated according to this principle (Feldman *et al*. 2000).

2.4.2. Serological Examinations and Assessment of Humoral Immune Response

The serum samples from each group were obtained on day twenty-eight and the vaccine immune response was evaluated by hemagglutination inhibition test according to this principle (Beard, 1980).

2.4.3. Assessment of Cell-mediated Immune Response

2.4.4. Lymphocyte Proliferation Assay

2.4.4.1. Separation of Lymphocytes (Burrells and Well, 1977)

The obtained heparinized blood samples were layered carefully on the surface of lymphocyte separation medium ficoll hypaque (1:1), centrifuged, and washed then re-suspended in 1 ml RPMI 1640 containing 10 % fetal calf serum.

2.4.4.2. Viable Lymphocyte Count (Hudson and Hay, 1980)

A hundred µL of 0.4 % trypan blue were added to the lymphocyte suspension, then mixed well and immediately

transferred to the haemocytometer. At least one-hundred viable lymphocytes were counted. The number of viable lymphocyte per ml was calculated according to the following equation:

Viable cell yield = (Viable cell count / Quadrants counted) X Dilution factor X

Hemocytometer factor X Current volume (mL)

2.4.4.3. Standardization of Lymphocyte Concentration for Blastogenesis :

According to the viable cell count, the viable lymphocytes were adjusted at a final concentration of 2 $x10^6$ cells/mL and suspended in RPMI medium containing 10 % fetal calf serum (FCS).

2.4.4.4. Preparation of Mitogen (non-specific mitogen): Phytohemagglutinin (Rai-el-Balhaa et al., 1985)

Phytohemagglutinin (PHA) was obtained as a powder and reconstituted in 5 mL RPMI medium. The required concentration could be made to 15 µL mL^{-1}.

2.4.4.5. Setting up of Lymphocyte Culture (Meky et al., 2001).

Sterile microtiter tissue culture flat bottom 96 well plates were used for cultivation of lymphocyte. The wells were used in triplicate manner; one contained 100 µL of suspended lymphocytes (2×10^6 cells) in 50 µL growth medium (RPMI + 10% FCS) and served as cell control. Another well contained 100 µL of suspended lymphocytes + 50 µL PHA (non-specific mitogen) as (15 µg / mL), and a third well group contained 150 µL of RPMI-1640 medium only. The total volume per well was adjusted to 150 µL. The plates were incubated at 37°C in a CO_2 incubator, lymphocyte transformation and blastogenesis were assayed after 48-72 hours using methyl tetrazolium bromide dye (MTT) assay.

Methyl tetrazolium dye (MTT) was added at $1/10^{th}$ of the total sample volume, and incubated at 37 ^oC in a 5 % CO_2 incubator for four hours. After incubation, the lysing buffer was added in 50 µL/ well. The plate was incubated overnight for complete cell transformation, then applied on a microplate reader ELx 800 UV (Bio-Tek) at 570 nm wave length.

2.5. Statistical Analysis

The obtained data were analyzed using SPSS software (SPSS, 2006) via applying analysis of one way ANOVA together with Duncan's Multiple Rang Test for testing the significant differences among the treated groups. Also, Student's T test for independent samples was used to detect the difference between the second and the third week results within the same group. The data were significant at (P<0.05).

3. Results

3.1. Culture of Aspergillus flavus

The culture of toxigenic *Aspergillus flavus* strain was maintained on potato dextrose agar plate; macroscopy revealed velvety, yellow to green or brown reverse goldish to red-brown figures (1 and 2). Microscopic morphology showed unbranched conidiophores, which were enlarged at the tip forming a swollen vesicle. Vesicles are completely covered with flask-shaped phialides, the phialides produce chains of mostly round conidia (figure 3).

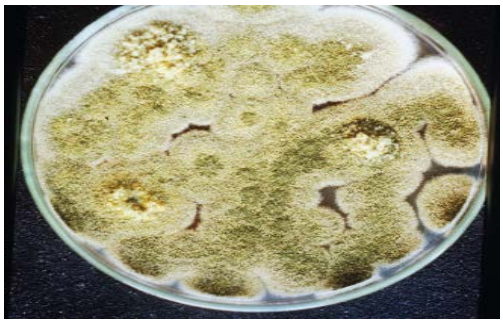

Figure 1. Potato dextrose agar plate showing growth of *A. flavus.*

Figure 2. Macroscopical morphology of *A. flavus* on potato derxtrose agar**.**

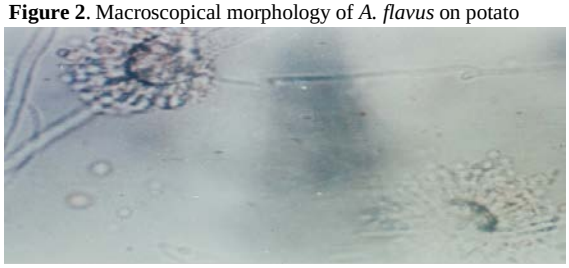

Figure 3. Microscopical morphology of *A. flavus* staining by lactophenol cotton blue (X 100) .

3.2. Qualitative Determination of Aflatoxin by (TLC)

The fungal extract was observed under UV light at 365 nm, and was matched with standard aflatoxin B1 as shown in figure 4.

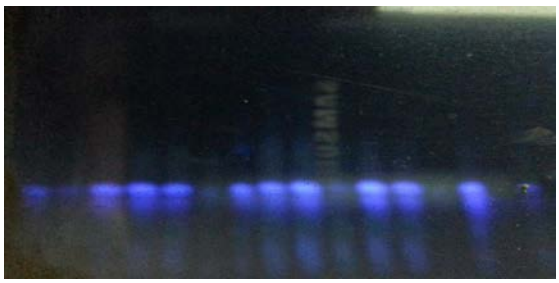

Figure 4. TLC for determining the toxigenicity of *Aspergillus flavus* strain.

3.3. Quantitative Determination of Aflatoxin B1 Using HPLC

Figure 5 shows two graphs (a, b). The first graph was for standards and the second one was for the samples. The quantification of aflatoxin B1 was determined from the area of the peak.

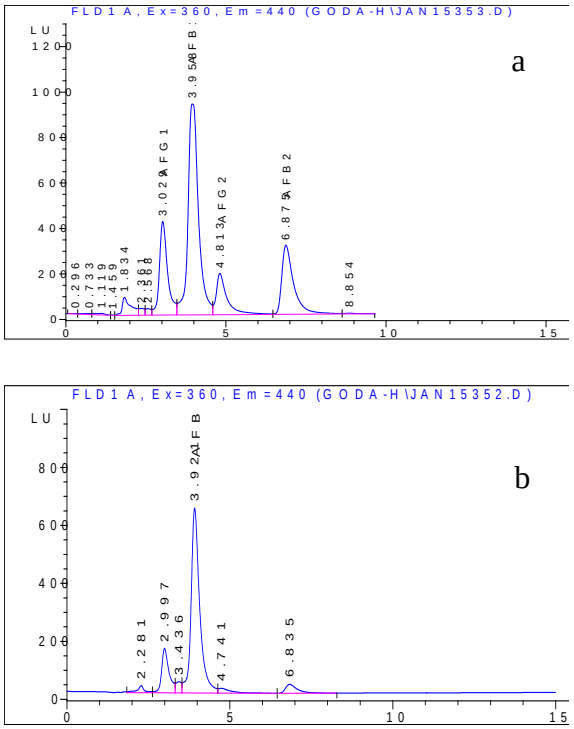

Figure 5. Typical high performance Liquid Chromatogram of aflatoxins (a) standards (AFG1,AFB1, AFG2 and AFB2); (b) Chromatogram of the chloroform extract of *Aspergillus flavus* strain.

3.4. Assessment of Immunological Parameters

3.4.1. Differential Leukocyte Counts

Differential leukocyte counts in different experimental groups of broilers after three weeks of feeding were represented in table 1. The lymphocyte percentage was significantly decreased in group three (lymphocytopenia) compared with the control negative group, while the heterophils percentage was in higher value, and these percentages were restored in group four. Regarding the monocytes percentage, all groups were significantly increased compared with the control negative group. The eosinophils in all groups were within the normal range.

Table 1. Differential leukocyte counts in different experimental groups of broilers after three weeks of exposure to the experimental diet.

Leukocytes %					
Groups	Heterophils	Eosinophils	Lymphocytes	Monocytes	H/L Ratio
1	30.20±0.75c	3.13±0.15a	62.50±0.40a	4.17±0.29c	0.48±0.02c
2	37.33±0.43b	3.13±0.15a	54.07±0.18b	5.47±0.12ab	0.69±0.01b
3	45.67±0.35a	2.80±0.06a	45.90±0.25c	5.63±0.20ab	1.00±0.01a
4	37.90±0.51b	3.13±0.09a	52.90±0.30b	6.07±0.15a	0.72±0.01b

The relationship between heterophils and lymphocytes was represented as the H/L ratio as shown in figure 6. There was a significant increase in H/L ratio in the aflatoxin feed group compared with other experimental groups after two and three weeks of exposure to the experimental diet.

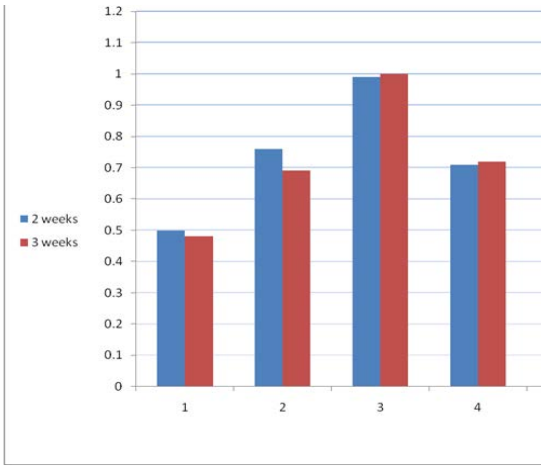

Figure 6. The relationship between heterophils and lymphocytes (H/L) ratio. Means within the same column carrying different superscripts are significantly differed at ($P \leq 0.05$).

3.4.2. Assessment of Humoral Immune Response

Haemagglutination inhibition test (HI) showed that there were significant differences of anti- NDV antibody titer among the treated groups after three weeks of exposure. The aflatoxin treated groups revealed a significant decrease compared with the negative control group. The supplementation of MOS to the aflatoxin-treated diet revealed a non-significant increase as shown in figure 7.

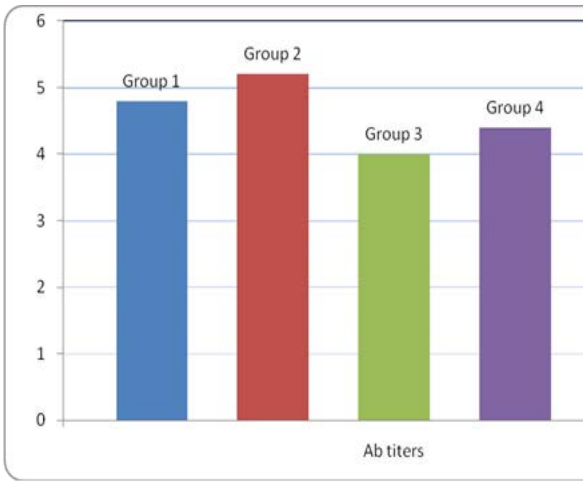

Figure 7. Antibody titers of the HI test against NDV vaccine. Means within the same column carrying different superscripts are significantly differed at ($P \leq 0.05$).

3.4.3. Assessment of Cell-mediated Immune Response "Lymphocyte Transformation Test"

Figure 8 represented the lymphocyte transformation after two weeks of feeding. The results showed that group three was decreased significantly compared with the two control groups. The control positive group was higher than the negative, while group four was similar to the control negative one.

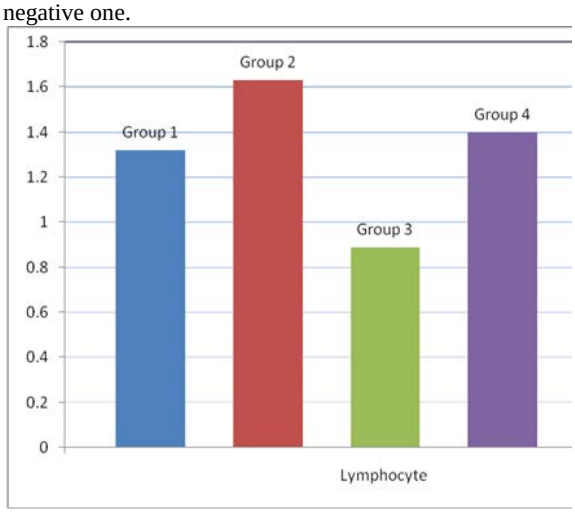

Figure 8. Lymphocyte transformation after two weeks of exposure to experimental diet. Means within the same column carrying different superscripts are significantly differed at ($P \leq 0.05$).

Figure 9 represented the Lymphocyte transformation after three weeks of feeding. The results still showed that group three was decreased significantly compared with the two control groups. The control positive group was higher than the negative one.

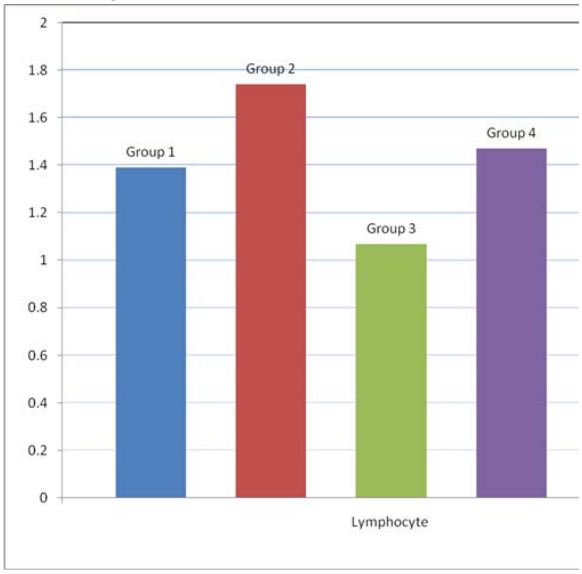

Figure 9. Lymphocyte transformation after three weeks of exposure to experimental diet. Means within the same column carrying different superscripts are significantly differed at ($P \leq 0.05$).

4. Discussion

Mycotoxins are one of the major factors affecting broiler's performance and immunity (Yunus *et al*., 2011). Therefore, the present study was carried out to investigate the effect of the aflatoxin (AFB1), and the efficacy of MOS on broiler's immune response parameters.

The results of differential leukocyte counts in table 1 demonstrated that the addition of AFB1 induced significant leukocytopenia, lymphocytopenia and heterophilia with a significant increase of monocytes. The increase of heterophil counts suggested that adding mycotoxins may elicit an inflammatory response in the chicks as was mentioned by (Keçeci *et al*., 1998). The decrease in the total leukocyte count, lymphocytes and the increase of heterophils can be attributed to the toxic effects of aflatoxins on circulating cells, sequestration of cells in tissues and / or the effects of aflatoxins on bone marrow and lymphoid tissues. The heterophil: lymphocyte ratio (H/L ratio) is a better indicator of stress in poultry representing changes in the number of circulating leukocytes, in particular, a pronounced heterophilia and lymphocytopenia (Maxwell, 1993). There was a significant increase in the H/L ratio represented in figure 6 in the aflatoxicated group compared with other experimental groups after three weeks of exposure to the experimental diet. The pervious findings are in accordance with the results obtained by (Andretta *et al*., 2012; Mohaghegh *et al*., 2017).

Mycotoxin-induced immunosuppression may be manifested as depressed T- or B-lymphocyte activity, suppressed antibody production and impaired macrophage/neutrophil-effector functions. As the immune system is primarily responsible for defense against invading organisms, so the suppressed immune function by aflatoxins may eventually decrease resistance to infectious diseases, reactivate chronic infections, and/or decrease vaccine and drug efficacy (Oswald *et al*., 2005).

Humoral immunity is mediated by antibodies released by B-cells into the bloodstream and effectively against extracellular antigens. Cell-mediated immunity (CMI) is based on specific antigen recognition by thymus derived T-lymphocytes, and specializes in the elimination of intracellular antigens. In birds, precursors of T-cell and B-cell originate in bone marrow, while the actual development of T-cells take place in the thymus and B-cells in the bursa of Fabricius (Surai and Dvorska, 2005). Concerning the assessment of humoral immune response through the estimation of antibody titers against NDV vaccine using HI test, the results in figure 7 revealed that there was a significant decrease in the titer. These results agreed with those obtained by (Otim *et al*., 2005) who reported that there was a significant reduction in the haemagglutination inhibition of ND antibody titer of 0.250 mg aflatoxin B1 per bird.

In this regard, the humoral immune response from broilers may decrease depending on the level and duration of the exposure to the toxins. These results were confirmed also by (Manafi *et al*., 2012) who reported that the biphasic nature of AFB1 on humoral immunity, humoral immune response might decline depending upon the dose and duration of exposure. Also, the results in figure 7 showed that supplementation of MOS could improve the immune response of broilers which indicates that MOS has immunomodulatory properties. These results agree with (Khalaji *et al*., 2011; Yalcin *et al*., 2013; and Mohaghegh *et al*., 2017).

Concerning the lymphocyte blastogenesis assay for the assessment of cellular immunity, the figures 8 and 9 revealed a significant reduction of the cell-mediated immune response in te aflatoxin treated group compared with the other control groups. These results exhibited the toxic and harmful effects of these mycotoxins on the cellular immunity. Similar results were obtained by (Girish and Smith, 2008, Jayaramu *et al*., 2012; and Anjorin and Cyriacus 2014).

This study revealed that MOS has the ability to detoxify aflatoxicosis in broilers through improving both humoral and cell-mediated immunity profile of broiler. The current results are in agreement with (Santin *et al*., 2003; Oguz and Parlat, 2004; and Pizzolitto *et al*., 2013) who confirmed the ability of MOS to reduce the deleterious effects of aflatoxin.

5. Conclusion

The present study concludes that the aflatoxin B1 caused toxic effects on humoral and cell-mediated immune responses of broilers with adverse lymphocytopenia and heterophilia. On the other hand, adding MOS to aflatoxin-treated ration led to the improvement of the immune health and the reduction of the toxic effects of AFB1on immune profile. Attention must be taken to avoid mycotoxicosis as it doesn't only cause harm to poultry, but also it has dangerous effects on human health because of its residual and cumulative nature.

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Screening Wild and Mutant Strains of *Aspergillus flavus* and *Aspergillus niger* Isolated from Plantain Stalks for Amylase Production

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Abstract

This study is conducted to determine the amylase activity of wild and mutant strains of *Aspergillus flavus* and *Aspergillus niger* isolated from plantain stalks. The isolation of the fungal species was carried out using standard microbiological methods. Strain improvement of the fungal isolates was carried out by exposing the wild fungal species to ultraviolet (UV) radiation at 240nm for ten, twenty, and thirty minutes. The amylase production from the wild and mutant strains was examined quantitatively while the effects of pH and temperature on the amylase activities of the wild and mutant strains were determined. Three mutant strains were obtained from each of *Aspergillus flavus* and *Aspergillus niger*. The wild and mutant strains of the fungal isolates showed variations in the amylase production. The amount of amylase produced by the fungal strains ranged from 2.849 mg/mL/min to 3.263 mg/mL/min of which the mutant strains of *Aspergillus niger*, exposed for ten minutes showed the highest amylase production. Furthermore, the amylase activities of the wild and mutant strains of the fungal species were sensitive to changes in pH and temperature. Amylase was optimally produced from all the fungal strains at pH 6 and 25^{o}C. This study has revealed the amylase producing potential of wild and mutant (irradiated) strains of *Aspergillus flavus* and *Aspergillus niger*. Therefore, the mutated strains of *Aspergillus flavus* and *Aspergillus niger* could be employed in the commercial production of amylase. Findings from this study are promising; however, further intensive studies are still needed on the improved strains as well as the purification and characterization of the enzyme.

Keywords: Amylase, Wild strains, Mutant strains, Plantain stalk, *Aspergillus flavus*, *Aspergillus niger*, Enzyme activity.

1. Introduction

Enzymes are potential biocatalysts for a large number of reactions and they form parts of the most important products needed to meet human needs in the areas of industrial, environmental and food biotechnology through microbial sources (Chaudhri and Suneetha, 2012). Over five-hundred industrial products are being made using enzymes (Kumar and Sing, 2013). Industrial enzymes are highly demanded, and there is a need for a solution through which they can be produced on a large scale.

In nature, microorganisms have been endowed with vast potentials. They produce an array of enzymes, which have been exploited commercially over the years. Microbes are known to be one of the largest and useful sources of many enzymes (Demain and Adrio, 2008). Microbial enzymes are known to play a crucial role as metabolic catalysts, leading to their use in various industries and applications. The advantages of microbial enzymes over plant and animal enzymes are that they are more active and stable. In addition, microorganisms represent an alternative source of enzymes because they can be cultured in large quantities in a short time by fermentation owing to their biochemical diversity and susceptibility to gene manipulation. The main concern of industries is to get new microbial strains with the ability to produce different enzymes to fulfil the current enzyme requirements (Singh *et al.,* 2016).

The most widely used thermostable enzymes are the amylases in the starch industry (Emmanuel *et al*., 2000) and the enzymes obtained from fungal isolates are much more useful in industries for bakery, starch conversion and biofuel production. Developing countries depend on enzyme production from fungi because of the non-fastidious nutritional requirement and ubiquitous nature of the fungal enzymes (Gigras *et al*., 2002). A distinguishing characteristic of all the commercial fermentation processes is the improvement of microbial strains for a higher enzymatic yield. Such improved strains can reduce the cost of the process, and may also impact some specialized desirable characteristics of the products (Steensels *et al*., 2014; Rowland, 1984).

A variety of agricultural and food processing waste substrates can be employed in the production of industrially important enzymes. Fruits and vegetable wastes constitute agricultural wastes that are considered as a major source of environmental pollution (Garg and

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Ashfaque, 2010). Agricultural wastes are the source of starch containing substrates and necessary carbon and nitrogen sources for microbial metabolism (Siddiqui *et al*., 2014). Various agricultural wastes such as millet starch, potato and wheat bran are widely used for the production of enzymes (Sajjad and Choudary, 2012). In this study, Plantain (*Musa paradisiaca*) stalk wastes which are under-utilized in general, are exploited for the production of enzymes.

Microorganisms can be easily manipulated using genetic engineering or other means. They can be subjected to strain improvement, mutations, and other such changes by which the production of enzymes can be optimized (Ajita and Thirupathihalli, 2014). Ultraviolet (UV) light has been shown to be lethal and mutagenic in a variety of organisms, including fungi. Ultraviolet irradiation was found to be the best for the improvement of strains such as *Aspergillus niger* for a maximum production of various enzymes (Kang *et al*., 1999). In recent years, attempts have been made for the over production of microbial enzymes by induced mutagens. Novel enzymes were developed by the combined use of microbial screening and rational protein engineering (Sarikaya *et al*., 2000). Such improved strains can reduce the cost of the process and may also possess some specialized desirable characteristics.

Enzymes of fungal origin were found to be more stable than the bacterial enzymes on a commercial scale. Developing countries depend on enzyme production from fungi because of the non-fastidious nutritional requirements and ubiquitous nature of the fungal enzymes (Gigras *et al*., 2002). Various forms of amylases are used to convert starch into different sugars. A variety of industries employ microbial amylase, cellulase and lipase which are used in textile, paper and detergent industries (Das *et al*., 2011; Tsukagoshi *et al*., 2001). α- Amylases, β amylase and glucoamylase are commonly produced by various species of *Aspergillus* and are used as sources of industrial amylases. The most widely used thermostable enzymes are the amylases in the starch industry (Emmanuel *et al*., 2000). In fact, amylase has received a great deal of attention due to the technological and economic significance (Asrat and Girma, 2018). The enzymes obtained from fungal isolates are much more useful in starch conversion, bakery and fuel alcohol production.

The aim of this study is to subject the fungal strains isolated from plantain stalks to random mutagenesis by UV light for strain improvement and to compare the enzyme activities of the wild and the mutants after exposure to the UV light.

2. Materials and Methods

2.1. Study Area/Sample Collection

The Federal University of Technology, Akure, Nigeria lies between longitude 5.1^{o} East and latitude 7.2^{o} North. Its population is estimated at twenty two thousand. A lot of wastes have been generated, amongst which are the plantain stalk wastes. A plantain stalk waste sample was collected at a dump site at Obakekere at the Federal University of Technology, Akure, Nigeria and was placed in a clean sterile polythene bag and transported to the Departmental Laboratory for microbiological analyses.

2.2. Isolation and Identification of Fungal Isolates

One gram **(**1g) of the plantain stalk was cut using a sterile razor blade and was placed in 10mL of distilled water. This was shaken properly inside a test tube to obtain the stock culture. Serial dilutions, plating on Sabouraud dextrose agar (SDA) incorporated with chloramphenicol, incubation and subculturing were done using standard techniques. The colonies were counted as spore forming unit (SFU/g), while the identification was based on the microscopic and macroscopic features of the hyphal mass, nature of the fruiting bodies, and the morphology of cells and spores.

2.3. Inoculum Preparation

The inoculum of the fungal isolates grown for ninety-six hours at 30°C on SDA medium slants was prepared by adding 10 mL of sterile distilled water, containing 0.1% (v/v) Tween 80 to the agar slant and was shaken vigorously. The spore suspension was adjusted to the spore concentration of 10^3 SFU/mL as the initial inoculum size (Ibrahim *et al*., 2012).

2.4. Mutant Generation by Ultraviolet Radiation

The method described by Bapiraju *et al*., (2004) was modified and employed in preparing the mutant fungal strains. The Petri plates of the wild fungal isolates were exposed to UV irradiation for ten, twenty, and thirty minutes at a distance of 10 cm in the dark (to prevent photo reactivation) from the center of germicidal lamp (240 nm) with occasional shaking. Afterwards, 1mL spore suspension was withdrawn from each labelled plates and was plated on SDA medium. The developed mutants were maintained on SDA slants at 4°C in the refrigerator until use. The developed mutants and wild parents were quantified for amylase production.

2.5. Quantification for Amylase Production

Soluble starch (1 %) was prepared in 0.02M sodium phosphate of pH 6.9 containing 0.006M NaCl. Thereafter, 0.2mL of the enzyme solution (extract) was added to 0.2mL of substrate (starch) and incubated at 25^{o}C for three minutes. Afterwards, 1mL of 3-5 Dinitro salicylic Acid reagents (DNSA) was added. The mixture was then heated in a water bath (100^{o}C) for five minutes. After heating, the mixture was cooled and 10 mL of distilled water was added, and then read in a colorimeter at 540 nm against a blank containing buffer without enzyme with the aid of a spectrophotometer. A calibration curve was made with maltose to convert the colorimeter reading to the unit of activity (Sohail, 2005).

2.6. Effect of Physical Factors on Amylase Activity

The effect of temperature on the amylase activity was determined by the method described by Adekunle *et al*., (2014). This was performed by incubating an aliquot of the enzyme with the substrate at temperatures ranging from 25^{o}C to 60°C for twenty minutes. The residual amylase activity was plotted against the different temperatures.

The effect of pH on the amylase activity was investigated within the pH range of 3 - 9 at room temperature. For the measurement of pH stability, the enzyme was incubated at room temperature for one hour in

buffers at different pH values, and the residual activity was determined. (Adekunle *et al*., 2014).

2.7. Statistical Analysis

The experiments were carried out in replicates of three, and the results were expressed as mean ± standard error of three values. Data obtained were subjected to the one Way Analysis of Variance (ANOVA), and means were compared using New Duncan's Multiple Range Test (SPSS version 16). Differences were considered significant at $P<0.05$.

3. Results and Discussion

3.1. Enumeration and Identification of Fungal Isolates

The result of the total fungal load of the plantain stalk is presented in Table 1. The total fungal load was 10 SFU/g. The morphological and microscopic tests carried out on the fungal isolates from plantain stalk confirmed the identity of the isolates to be *Aspergillus niger* and *Aspergillus flavus* (Table 2).

Table 1. Enumeration of fungal isolates

Sample	Dilution factor	Total Count (SFU/g)	Species Description of fungal isolate
Plantain stalk	10^{-2}	10	Green and black colonies

Table 2. Cultural and morphological characteristics of the isolated fungal species.

Cultural characteristics	Microscopic characteristics	Organism
Brown mycelial growth	An upright conidiophore that terminates in a swelling, bearing phialides at the apex radiating from the entire surface: conidia are one celled. Spores are black.	*Aspergillus niger*
Greenish yellow mycelia growth and fully extended from the growth medium, conidiophores upright, simple terminating.	Branched hyphae and septate conidiophores, long upright aseptate and unbranched, no columella, terminate into globose vesicles surface contain many flask-shaped phialides with chains of conidia. Has yellowish green spores	*Aspergillus flavus*

3.2. Amylase Production by Wild and Mutant Fungal Isolates

Amylase production by the wild and mutant fungal isolates ranged between 2.849 and 3.263 mg/mL/min (Table 3).

When comparing the amylase activity of wild strains and the mutant strains exposed to the first dose of UV radiation, it was observed that the amylase production increased in the mutant strains. The amylase activity was found to be higher in mutants *Aspergillus flavus* exposed to UV for ten minutes (AFUV10) (3.243 mg/mL/min) and *Aspergillus niger* exposed to UV for ten minutes (ANUV10) (3.263 mg/mL/min). However, a decrease in amylase activity was observed in the mutant strains with prolonged exposure to UV radiation.

Aspergilus niger strains	Amylase quantity (mg/mL/min)	*Aspergillus flavus* strains	Amylase quantity (mg/mL/min)
ANW	3.162 ± 0.061^{c}	AFW	2.960 ± 0.030^{b}
ANUV10	3.263 ± 0.002^{d}	AFUV10	3.243 ± 0.004^{d}
ANUV20	3.246 ± 0.011^{d}	AFUV20	3.186 ± 0.002^{cd}
ANUV30	3.195 ± 0.003^{cd}	AFUV30	2.849 ± 0.003^{a}

Table 3. Amylase activity of wild type and UV mutant strains of *Aspergillus niger* and A*spergillus flavus.*

3.3. Effect of Temperature on Amylase Activity of Fungal Isolates

The effect of tempertaure on the wild and improved strains of *Aspergillus niger* and *Aspergillus flavus* is presented in Figure 1. The amylase activities of the fungal strains was found to be temperature-dependent. The fungal strains except for the improved strain of *A. niger* exposed to UV for ten minutes (ANUV10) produced amylase optimally (2.84 to 3.23 mg/mL/min). *A.niger* exposed to UV for thirty minutes (ANUV30) displayed the highest amylase activity (3.23mg/mL/min) while ANUV10 displayed the least amylase activity (2.84 mg/mL/min).

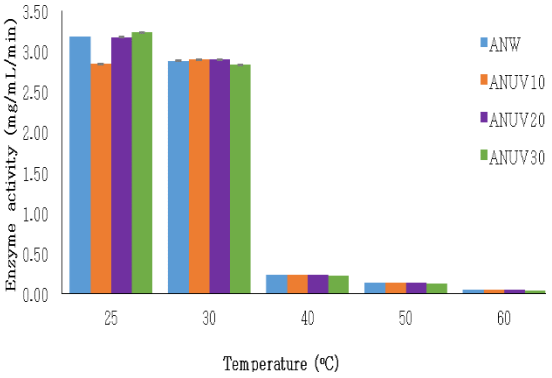

Figure 1. Effect of temperature on amylase activities of wild and mutant strains of *A. niger*. ANW: wide strain of *A. niger*. ANUV 10*: A. niger* exposed to ultraviolet light at 10 minutes ; ANUV20: *A. niger* exposed to ultraviolet light at 20 minutes; ANUV 30: *A. niger* exposed to ultraviolet light at 30 minutes.

Figure 2 also shows the effect of temperature on amylase activities of wild and mutant strains of *Aspergillus flavus*. The amylase activities of the fungal strains were found to decrease upon increasing temperature. The organisms produced varying amylase activities (0.04 to 3.24 mg/mL/min) within the temperature range of 25 ^{o}C to 60^{o}C. The optimal enzyme activity was at 25^{o}C with *A. flavus* exposed to 10 (AFUV10) and 20 (AFUV20) displaying the best activity.

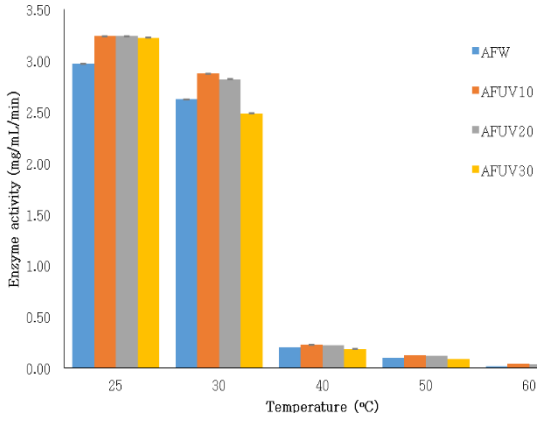

Figure 2. Effect of temperature on amylase activities of wild and mutant strains of *Aspergillus flavus*. AFW: wide strain of *A. flavus*. AFUV10*: A. flavus* exposed to ultraviolet light at 10 minutes. AFUV20: *A. flavus* exposed to ultraviolet light at 20 minutes. AFUV30: *A. flavus* exposed to ultraviolet light at 30 minutes.

3.4. Effect of pH on Amylase Activity of Fungal Isolates

The effect of pH on the amylase activity of the *A.niger* strains is presented in Figure 3 . The fungal strains were found to display pH-dependent enzyme activities. The enzyme activity of the fungal strains was found to be low (0.12 to 0.21 mg/mL/min) at pH 3 and pH 4. A sharp increase in the amylase activity was displayed by the strains of *A.niger* at pH 5 (2.23 to 2.30 mg/mL/min) and pH 6 (3.19 to 3.24 mg/mL/min). However, further increase in the pH values led to a sharp decline in the enzyme activity (0.32 - 0.14mg/mL/min**).** Similar observation was also made in the amylase activity of *A. flavus* strains when exposed to varying pH (Figure 4). Strains of *A. flavus* produced amylase optimally at pH 6 (2.84 to 3.23 mg/mL/min**).** The improved strain of *A. flavus*, AFUV10 displayed the highest enzyme activity (3.23 mg/mL/min) at pH 6.

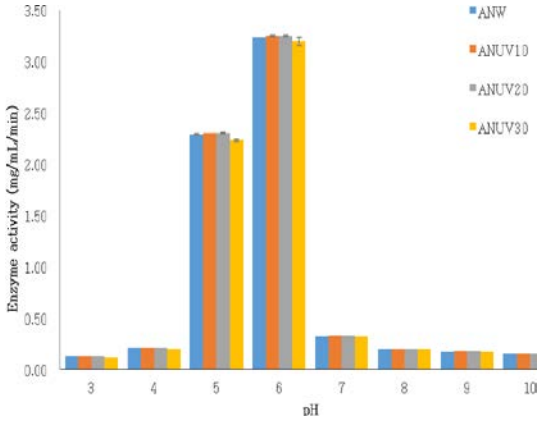

Figure 3. Effect of pH on amylase activities of wild and mutant strains of *A. niger*. ANW: wide strain of *A. niger*. ANUV10*: A. niger* exposed to ultraviolet light at 10 minutes. ANUV20: *Aspergilus niger* exposed to ultraviolet light at 20 minutes. ANUV30: *Aspergilus niger* exposed to ultraviolet light at 30 minutes.

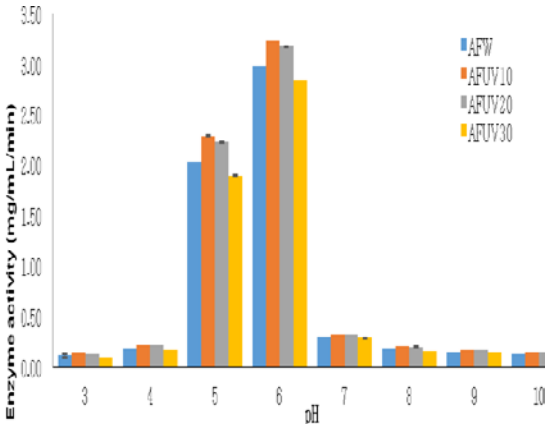

Figure 4. Effect of pH on amylase activities of wild and mutant strains of *A. flavus*. AFW: wide strain of *A. flavus*.. AFUV10*: A. flavus.* exposed to ultraviolet light at 10 minutes. AFUV20: *A. flavus.* exposed to ultraviolet light at 20 minutes. AFUV30: *A. flavus.* exposed to ultraviolet light at 30 minutes.

Results from the study showed two fungal species, *Aspergillus flavus* and *Aspergillus niger* isolated from the plantain stalks. These fungal species have been implicated in the biodegradation of agro-wastes and the production of industrially important enzymes (Thangaratham and Manimegalai, 2014). Hence, their presence on the plantain stalk indicates the biodegradation of the plantain stalk. The results of the current study also showed *Aspergillus flavus* and *Aspergillus niger* producing amylase at varying degrees. This observation is consistent with the findings of Oseni (2011) and El-Tablawy (2014). While studying the protease activity of some fungal isolates, Oseni (2011) suggested that protease production was directly linked to the organism involved, and the effectiveness and sustainability of the culture medium used. El-Tablawy, (2014) also observed different fungal species isolated from medicinal plants to produce extracellular enzymes at varying degrees.

Furthermore, *A. flavus* and *A. niger* mutant strains after exposure to 10 mins of ultraviolet radiation were found to produce higher amount of amylase in comparison with the wild strain. This suggests that strain improvement through irradiation by UV could lead to improved amylase production by the isolates. Thymine and cytosine are reportedly sensitive to modification when exposed to UV radiation as it can lead to the production of thymine dimers that can distort the DNA helix and block further replication (Sambrook and Russell, 2001). Several authors have reported enzyme production by microorganisms when exposed to UV radiation. For instance, a higher cellulase activity by UV mutant strains of *Trichoderma reesei* was reported by Shahbazi *et al*., (2014). However, longer exposure of the fungal strain led to a decline in the amylase production. This implies that longer exposure of the strains to UV radiation might be rather lethal than beneficial to the organisms in terms of amylase production.

The catalytic activities of enzymes are reportedly temperature and pH-sensitive (Pathak *et al.*, 2014). The use of optimal temperature for enzyme activity is an indispensable parameter especially in starch-processing industries (Sohail, 2005). The enzyme produced by the fungal strains used for this study displayed varying

thermostability, acted optimally at temperature of 25°C, and displayed the least thermostability at 60°C. This finding corroborates with the results of Alva *et al*., (2007) who reported similar thermostability range for amylase produced by *Aspergillus* species.

The findings of the current study showed that amylase produced by each fungal strain is pH- sensitive. For instance, amylase produced by each fungal strains was optimally active at acidic or near neutral conditions (pH 5 to 6). This indicates that the fungal strain prefers slightly acidic or neutral pH for its activity. This result concurs with the findings of Dutta *et al*. (2016). In their findings, they observed amylase produced by fresh water zooplankton, *Heliodiaptomus viduus*, had more than 50 % activity between pH 4.6 and 6.8. A similar observation was also made by Aydoğdu *et al*., (2012) who noted that the amylolytic activity of the fungal species used for the experiment falls between pH 4 and 6, while Asrat and Girma, (2018) observed a maximum activity at pH 6.0 in the production of the amylase enzyme by *Aspergillus niger* FAB- 211.

The ANOVA was used to compare the difference between the fungal isolates exposed to UV radiation for ten, twenty, and thirty minutes. Difference was considered significant at the 95 % confidence level ($p < 0.05$) (Table 3).

4. Conclusion

The current study has revealed the amylase producing potential of wild and irradiated strains of *Aspergillus flavus* and *Aspergillus niger*. The results from the study also showed improvement in amylase production after exposure to UV radiation after ten minutes. Furthermore, the amylase was seen to be optimally active at pH 6 and at 25°C. Therefore, these strains could be employed in the commercial production of amylase, and could help solve one of the challenges faced in the industrial setup where amylase utilization is high. The cost of disposing the plantain stalk will be reduced, and the environmental pollution arising from the decomposition of the wastes will also be reduced as well.

Findings of the current study are promising, however, there is more need for intensive further studies on the improved strains.

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Additive Effects of Physical Exercise and Environmental Enrichment in Attenuating Alterations in the Hippocampal Neuronal Morphology of Adult Wistar Rats Induced by Prenatal Inflammations

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Abstract

The present study is aimed at elucidating the additive effects of treadmill running exercises and environmental enrichment (E.E) during adolescence in ameliorating the alterations in the dendritic morphology of hippocampal CA3 neurons induced by prenatal inflammations. Pregnant Wistar dams were injected intraperitoneally with either 0.5ml of saline (control group) or lipopolysaccharide (LPS group) (0.5mg/kg), from the embryonic day fourteen till delivery, on alternate days. Following parturition, pups were allocated into following groups [n=6/group]: (1) Control, (2) LPS, (3) LPS-exercise, (4) LPS-environmental enrichment and (5) LPS-exercise-environmental enrichment. On postnatal days fifteen to sixty, rats of the groups three, four and five were subjected either to treadmill running exercises or environmental enrichment or the combination of the two, respectively. On PND (postnatal day) sixty-seven, animals were euthanized, brains were carefully dissected out, and impregnated with modified Golgi-cox stain. Dendritic arborization of CA3 neurons in hippocampus was traced by camera lucida and analysed by Sholl's method. The young adult rats of LPS- environmental enrichment -exercise group showed a significant enhancement in dendritic arborization of CA3 hippocampal neurons, compared with other groups. Being reared in an intricate and enriched environment supported by treadmill exercises during adolescence enhances the dendritic arborization of hippocampal CA3 neurons that were exposed to LPS-induced prenatal inflammations. This study investigates the effects of using these methods in combination rather than administering either treadmill exercise, or environmental enrichment each on its own.

KeyWords: Prenatal inflammation, Lipopolysaccharide, Physical exercise, Enriched environment, dendritic arbor.

1. Introduction

Exposure to various insults during gestation such as stress, infection, malnutrition, etc. may cause adverse chronic changes in behavior, neuroendocrine responses, and cognition of the offspring (Kohman *et al.,* 2008). Early life experience can profoundly impact the shaping of the physiological and psychological health of an individual (Kohman *et al.,* 2008). Epidemiological evidence shows an increased risk of developing several neuropsychiatric disorders such as schizophrenia (Brown, 2012), autism (Ciaranello and Ciaranello, 1995), mental retardation (McDermott *et al.,* 2000), and cerebral palsy (Hermansen and Hermansen, 2006), in addition to preterm birth, following prenatal inflammations induced by infections. Studies have investigated the effects of prenatal stress or immune challenge on the alterations of neuronal cytoarchitexture and on behavior or on both.

Asymptomatic infections, either bacterial or viral, during pregnancy that remain undiagnosed can lead to severe deleterious complications even intrauterine fetal death. Systemic administration of lipopolysaccharide (LPS), a cell-membrane component of gram negative bacteria, is a commonly accepted model to challenge the immune system during gestation leading to induction of inflammatory chemical mediators-the cytokines (Cui *et al.,* 2009). Maternal infection during gestation is thought to affect the developing fetal brain by inducing the proinflammatory cytokines in both compartments i.e. fetal and maternal compartments (Golan *et al*., 2005, Ashdown *et al*., 2006). Pregnant rats injected with LPS showed

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induction of interleukin-1 beta (IL-1β), interleukin- 6 (IL6) and Tumor-necrosis factor- alpha (TNFα) in the amniotic fluid, maternal and fetal sera. In humans, the prenatal exposure to lipopolysaccharide during gestation takes place as part of bacterial vaginitis (Ling *et al.*, 2009). In women aged between 25-40 years, *E.coli* is the most common (95 % approximately) etiological factor resulting in urinary tract infections (Faro and Fenner, 1998).

Early postnatal life events and experiences during the adolescent period play a crucial part in the behavioral development of an individual during adulthood. In the past decade, studies have documented the abilities of physical exercise (PE) in alleviating cognitive decline due to senescence, increasing hippocampal volume, promoting the hippocampal neurogenesis, reducing apoptosis in the hippocampus and preventing the neurodegenerative diseases (García-Capdevila *et al*., 2009, van Praag *et al*., 2005, Kirk-Sanchez and McGough, 2014, Kim *et al*., 2010, Bherer *et al.*, 2013), as well as enhancing learning and memory processes, for example, the abilites in Morris water Maze (Creer *et al.*, 2010), radial arm maze (Anderson *et al.*, 2000) in addition to facilitating long-term potentiation (van Praag *et al*., 1999b).

Enriched Environment (EE) includes housing the animals in a complex variety of sensory-motor stimuli like a tunnel, swings, toys, running wheels, and social interaction with cage-mates (Lambert *et al.*, 2005). Studies have demonstrated that EE can increase dendritic arborization (both number of spines and dendritic branching), the size of the neuronal cell body and the level of neurotransmitters (Ickes *et al*., 2000, Rampon *et al*., 2000b). Middle- and old-aged rats and mice housed in intricate enriched conditions showed a reduction in age-related impairments in various types of learning and memory performance *(*Frick and Fernandez, *2003*, Kempermann *et al.*, 1998). Evidence from several studies confirms the beneficial role of PE and EE in various models of disease in rodents. However, the beneficial effects of PE and EE during the adolescent period in prenatal immune challenge models still need to be thoroughly studied. Similarly, studies of the additive effects of treadmill exercise along with the enriched housing conditions in reversing the alterations in neuronal morphology induced by prenatal LPS exposure are scarce. Thus, this study was conducted to investigate the additive effects of treadmill running exercises followed by enriched housing conditions in reversing the alterations in the neurons hippocampal region induced by prenatal LPS inflammations.

2. Materials and Methods

2.1. Animals

The study protocol was approved by the Institutional Animal Ethics Committee (IAEC), Manipal Academy of Higher Education, prior to the commencement of the experiment (No: IAEC/ KMC/ 01/2015). Maintenance of animals was performed according to the prescribed guidelines of the Committee for the Purpose of Control and Supervision of Experimental Animals (CPCSEA), Govt. of India. Adult female Wistar rats (n=24), aged three-months were housed in sterile cages with paddy husk as bedding, under standard laboratory conditions (22 ± 2°C temperature and 50 ± 5 % humidity) with a twelve-hour light/dark cycle. Water and food access to animals were *ad libitum*.

A pair of adult nulliparous female rats were caged with an adult male rat, pregnancy positivity was confirmed by the presence of sperms in the vaginal smear examined daily, and was considered as embryonic day '0' (E0). After random allocation either to control (n=6) or LPS group (n= 18), the pregnant dams were intraperitoneally (i.p) administered either with non-pyrogenic, sterile saline (0.5mL) or LPS (0.5mg/Kg, *E.coli* serotype 0111: B4 Sigma-Aldrich) respectively, from E14 till parturition on alternate days. After parturition, the pups were randomly assigned to groups (n=6/group), all offspring were raised by their biological mother, and the male offspring were used for the experiment. The pups born to the LPS mother were further sub-grouped as follows, (a) LPS, (b) LPS Exercise (LPS-Ex), (c) LPS Environmental Enrichment (LPS- EE) and (d) LPS Environmental Enrichment and Exercise (LPS- EE-Ex), in addition to the control group pups.

2.2. Treadmill (running) Exercise

Rats of the LPS-Ex and LPS-EE-Ex groups were subjected to treadmill running exercises, fifteen minutes/ per day [five sessions, three minutes/ per session with an intersession interval of four-five minutes approximately] from PNDs fifteen till PND sixty. The treadmill was equipped with horizontal motor-driven, five-parallel runways (IIITC Life Science, CA, USA. Model 805, Series 800). To avoid stressful conditions, the running speed was gradually increased from 1.5 meter/min (on PND 15) to 10.9 meters/min (on PND 25). The running speed of 10.9 meters/min was constantly maintained from PND twenty-five till PND sixty (Toy *et al*., 2014).

2.3. Environmental Enrichment

From PNDs fifteen to sixteen, four hours daily, the LPS-EE and LPS-EE Ex rats were subjected to environmental enrichnment. They were housed in a large sterile plastic cage, (120cm x 100cm x 100cm) with husk bedding, containing hard plastic tunnels, raised metal platform, ladder, objects of various sizes (metal balls, toys) and a steel swing [the cage was not provided with a voluntary running wheel]. The objects in EE cage were changed on alternate days.

2.4. Modified Golgi-Cox Staining

On PND sixty-seven, the young adult rats were euthanized following deep anaesthesia by an intramuscular injection of ketamine (100mg/kg body weight). The whole brain was carefully removed without transcardial perfusion, and was immersed in Golgi-Cox reagent for three to four weeks. The modified Golgi-cox solution was prepared as follows: five parts of 5 % potassium dichromate mixed with five parts of 5 % mercuric chloride, to this four parts of 5 % potassium chromate diluted with five parts of distilled water was added slowly with continuous stirring (Suvarna *et al.*, 2013). This solution was left undisturbed for four hours, and was filtered before use. After three weeks, each brain tissue was mounted on microtome tissue holder chucks with fevikwik glue. The tissue was coronally sliced at a

thickness of 180µm using a sledge microtome (Spencer sledge microtome) and collected in distilled water. Following treatment with the 5 % sodium carbonate solution for fifteen–twenty minutes, the sections were subjected to dehydration in ascending grades of alcohol, and were cleared in xylene. Finally, the sections were mounted with distyrene plasticizer xylene [DPX] on gelatin pre-coated glass slides, and cover slipped.

2.5. Tracing and Analysis of Hippocampal Neuron

The current study examined the two major morphological features - dendritic branching points and dendritic intersections as an indicator of dendritic arborization of a neuron of the hippocampal CA3 region. Golgi-Cox stained CA3 pyramidal neurons (six-eight neurons/animal) of the hippocampus was observed under 100x magnification and drawn on a plain white sheet of A4 size with the assistance of camera lucida attached microscope. The Sholl's method of the concentric circle was followed to quantify dendritic arborization. The researchers have used Sholl's grid with five concentric circles drawn at a calibrated (calibration equivalent to 20µm) distance of 2cm between each circle (Figure 1) (O'Neill *et al*., 2015). For neuronal analysis, Sholl's grid drawn transparent sheet was kept on a traced neuron in a manner that the center of the soma of traced neuron coincides with the mid-point of the smallest circle. A number of dendritic branching points, at the apical and basal level, between the concentric circles were noted. The number of dendrites crossings per radius (dendritic intersections) was counted. The analysis was carried after blinding the slides with codes to minimize any bias by the experimenter.

2.6. Statistical Analysis

Statistical analysis was performed using SPSS 16.0 for windows. Mean value for all neurons of each animal was calculated and then mean group difference in dendritic branching points and dendritic intersections were analyzed. One-way Analysis Of Variance (ANOVA) followed by Tukey's post-hoc test were used to analyse the data. A statistical significant level of $p<0.05$ was considered. The data in the graph are expressed as the mean ± SEM.

3. Results

In the Golgi-cox stained sections of control, LPS, LPS-Ex, LPS-EE and LPS-EE-Ex groups, to ascertain the spatial dispersion of dendritic branches in the apical as well as the basal regions in relation to the neuronal cell body, the mean number of the dendritic branching points and dendritic intersections per concentric sphere was analyzed for each group.

3.1. Dendritic Branching Points in the Apical Region

Analysis of apical branching points of CA3 pyramidal neurons revealed that there was a significant difference in the mean dendritic branching points among the groups between the circles as shown in Table 1; Figures 1- 4.

Table 1. Apical dendritic branching points of CA3 pyramidal neurons.

Sholl's Concentric Circle level (µm)	ANOVA "F" value; level of significance	Tukey's Post-hoc comparison	
		Groups	Level of significance
20-40	F (4, 25) = 4.338;$p<0.01$	LPS Vs LPS-Ex	$p<0.01$
40-60	F (4, 25) = 7.969 ;$p<0.001$	Control Vs LPS LPS Vs LPS-EE-Ex	$p<0.001$ $p<0.01$
60-80	F (4, 25) = 6.350; $p<0.01$	Control Vs LPS LPS Vs LPS-EE LPS Vs LPS-EE-Ex	$p<0.05$ $p<0.05$ $p<0.001$
80-100	F (4, 25) = 7.614; $p<0.001$	Control Vs LPS LPS Vs LPS-Ex LPS Vs LPS-EE LPS Vs LPS-EE-Ex	$p<0.05$ $p<0.05$ $p<0.01$ $p<0.001$

The analysis indicates that young adult rats of the LPS group showed reduced dendritic branching. Whereas, the rats that were exposed prenatally to LPS and were subjected to postnatal treadmill exercises followed by environmental enrichment (LPS-EE-Ex group) during adolescent age, showed increased dendritic branching points in the apical region of CA3 pyramidal neurons.

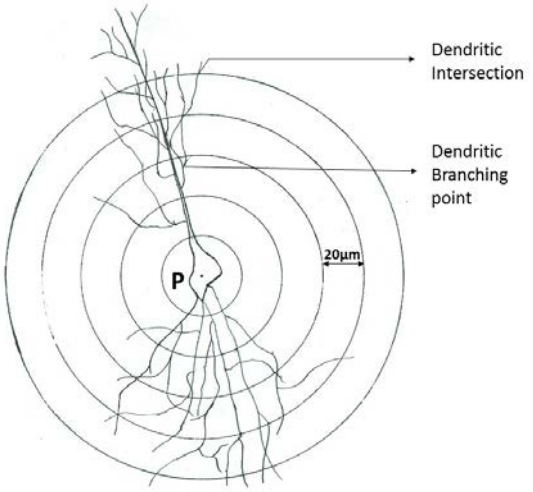

Figure 1. Representative image of camera lucida tracing of modified Golgi-Cox stained neuron of hippocampal CA3 region. The superimposed Sholl's grid containing successive concentric circles placed at an interval of 20µm from each circle. Letter 'P' indicates perikaryon.

3.2. Dendritic Intersections in the Apical Region

Analysis of apical intersections of dendrites of CA3 pyramidal neurons of groups showed that there was a significant difference in the mean number of dendritic intersections at the various radii level between the groups as shown in Table 2; Figures 1- 5.

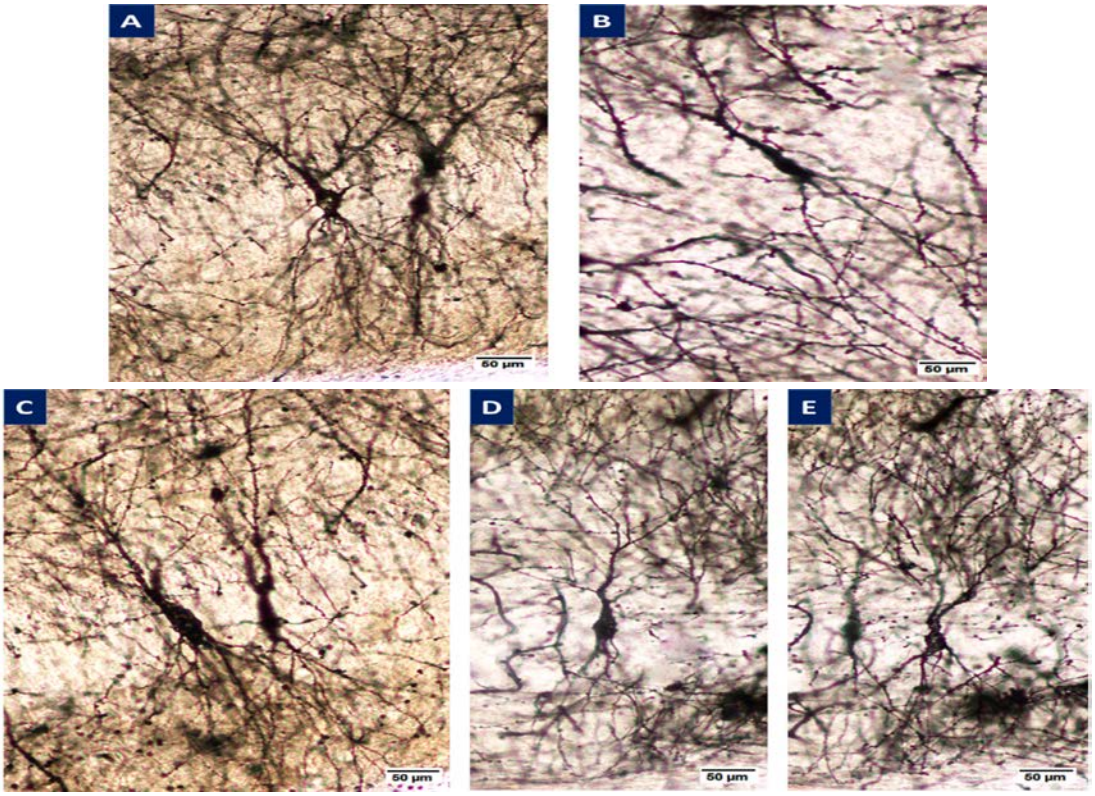

Figure 2. Representative photomicrographs of neurons of hippocampal CA3 region, stained with modified Golgi-Cox stain. Alphabet denotes: A- Control group; B- LPS group; C- LPS Ex group; D- LPS EE group and E- LPS EE Ex group. Photograph captured under 100x magnification

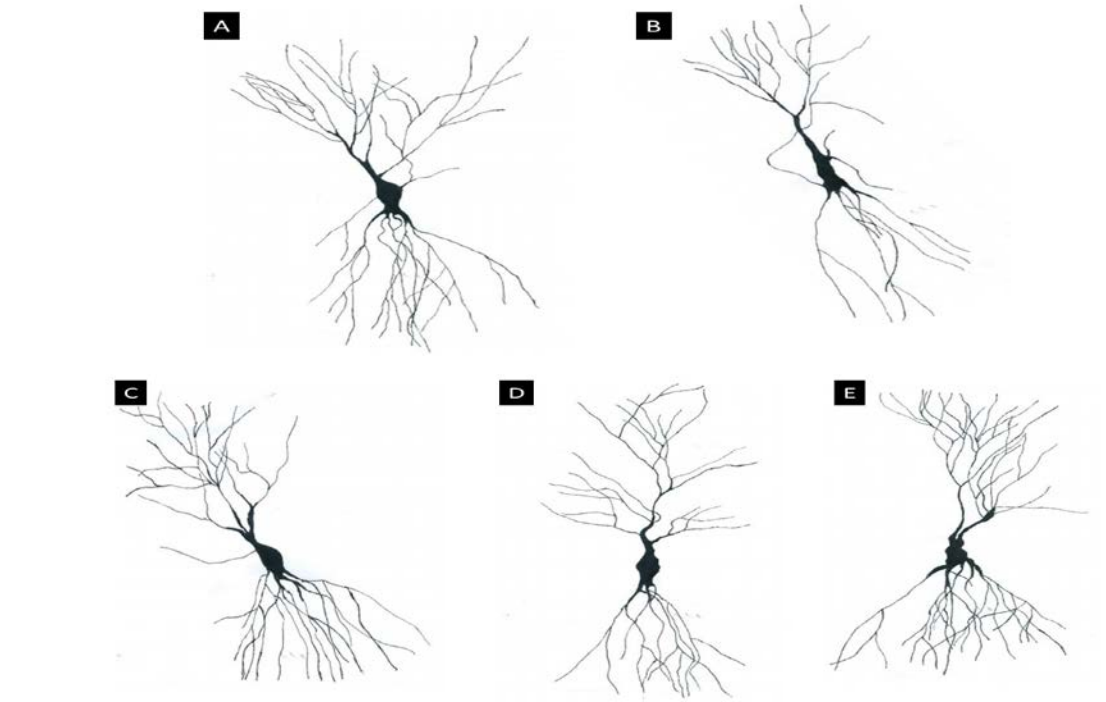

Figure 3. Representative images of camera lucida tracings of modified Golgi-Cox stained neurons of hippocampal CA3 region. Alphabet denotes: A- Control group; B- LPS group; C- LPS Ex group; D- LPS EE group and E- LPS EE Ex group. Images drawn under 100x magnification.

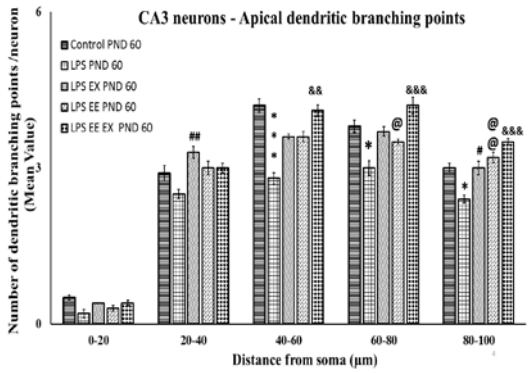

Figure 4. Effects of prenatal exposure to LPS and adolescent running exercise or being reared in a complex and enriched environment (EE) or a combination of the two methods of exercise and EE, on apical dendritic branching points of CA3 neurons. Symbols indicate levels of significance *p<0.01 and ***p<0.001- LPS Vs Control: && p<0.01 and &&& p<0.001 - LPS Vs LPS-EE-Ex; ## p<0.01 and # p<0.05- LPS Vs LPS-Ex; @ p<0.05 and @@ p<0.01- LPS Vs LPS-EE. Each bar represents mean ± SEM.

Table 2. Apical dendritic intersections of CA3 pyramidal neurons.

Sholl's Concentric Circle level (µm)	ANOVA "F" value; level of significance	Tukey's Post-hoc comparison	
		Groups	Level of significance
40	F (4, 25) = 6.413;p<0.01	Control Vs LPS	p<0.05
		LPS Vs LPS-Ex	p<0.01
		LPS Vs LPS-EE	p<0.05
		LPS Vs LPS-EE-Ex	p<0.05
60	F (4, 25) = 20.492;p<0.001	Control Vs LPS	p<0.001
		LPS Vs LPS-Ex	p<0.001
		LPS Vs LPS-EE	p<0.001
		LPS Vs LPS-EE-Ex	p<0.001
80	F (4, 25) = 10.975;p<0.001	Control Vs LPS	p<0.001
		LPS Vs LPS-Ex	p<0.01
		LPS Vs LPS-EE	p<0.05
		LPS Vs LPS-EE-Ex	p<0.001
100	F (4, 25) = 17.056;p<0.001	Control Vs LPS	p<0.001
		LPS Vs LPS-Ex	p<0.001
		LPS Vs LPS-EE	p<0.001
		LPS Vs LPS-EE-Ex	p<0.001

Analysis indicates that young adult rats of LPS group showed reduced dendritic intersections at the different radii of Sholl's grid. Whereas, the rats that were exposed prenatally to LPS and subjected to postnatal treadmill exercise followed by environmental enrichment (LPS-EE-Ex group) during adolescent age, showed increased dendritic intersection in the apical region of CA3 pyramidal neurons.

3.3. Dendritic Branching Points in the Basal Region

The basal branching points analysis of CA3 pyramidal neurons revealed that there was a significant difference in the mean dendritic branching points among the groups between the concentric circles. (Table 3; Figures 1, 2, 3 and 6).

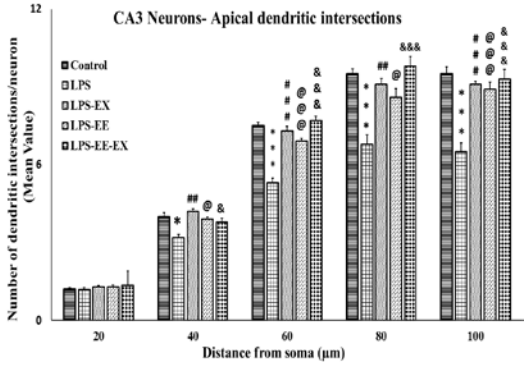

Figure 5. Effects of prenatal exposure to LPS and adolescent running exercise or being reared in a complex and enriched environment (EE) or a combination of the two methods of exercise and EE, on apical dendritic intersections of CA3 neurons. Symbols indicate levels of significance *p<0.01 and ***p<0.001- LPS Vs Control; ## p<0.01 and ### p<0.05- LPS Vs LPS-Ex; @ p<0.05 and @@@ p<0.001- LPS Vs LPS-EE; & p<0.05 and &&& p<0.001 - LPS Vs LPS-EE-Ex. Each bar represents mean ± SEM.

Table 3. Basal dendritic branching points of CA3 pyramidal neurons.

Sholl's Concentric Circle level (µm)	ANOVA "F" value; level of significance	Tukey's Post-hoc comparison	
		Groups	Level of significance
20-40	F (4, 25) = 3.625;p<0.05	Control Vs LPS	p<0.01
40-60	F (4, 25) = 6.818 ;p<0.01	Control Vs LPS	p<0.001
		LPS Vs LPS-EE-Ex	p<0.05
60-80	F (4, 25) = 4.749; p<0.01	Control Vs LPS	p<0.01
		LPS Vs LPS EE Ex	p<0.05
80-100	F (4, 25) = 5.055; p<0.01	Control Vs LPS	p<0.01
		LPS Vs LPS EE Ex	p< 0.05

Analysis indicates that prenatal exposure to LPS reduced the basal dendritic branching points of CA3 neurons of young adult rats (LPS group). Whereas, the young adult rats (PND 67) that were exposed to prenatal LPS and subjected to postnatal treadmill exercise followed by environmental enrichment (LPS-EE-Ex group) during adolescent age, showed increased dendritic branching points in the basal region of CA3 pyramidal neurons.

3.4. Dendritic Intersections in the Basal Region

The analysis of basal dendritic intersections of CA3 pyramidal neurons of PND sixty groups revealed that there was a significant difference in the mean dendritic intersections of dendrites among the groups at the different radii of the Sholl's grid. (Table 4; Figures 1, 2, 3, and 7).

The young adult rats that were exposed to LPS induced inflammation (LPS group) showed reduced basal dendritic intersections at the different radii of Sholl's grid. Whereas, the rats that were exposed prenatally to LPS, and were subjected to postnatal treadmill exercise followed by environmental enrichment (LPS-EE-Ex group) during adolescent age, showed increased dendritic intersection in the basal region of CA3 pyramidal neurons.

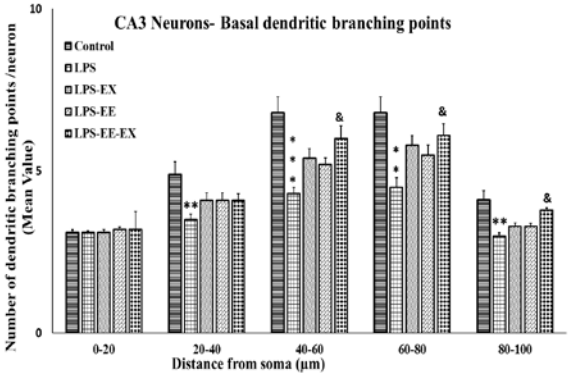

Figure 6. Effects of prenatal exposure to LPS and adolescent running exercise or being reared in a complex and enriched environment (EE) or the combination of the two, i.e., exercise and EE, on basal branching points of CA3 neurons. Symbols indicate levels of significance ***p*<0.01, ***p*<0.01 and ****p*<0.001- LPS Vs Control; & *p*<0.05 - LPS Vs LPS-EE-Ex. Each bar represents mean ± SEM.

Table 4. Basal dendritic intersections of CA3 pyramidal neurons.

Sholl's Concentric Circle level (µm)	ANOVA "F" value; level of significance	Tukey's Post-hoc comparison	
		Groups	Level of significance
40	F (4, 25) = 8.421;*p*<0.001	Control Vs LPS	*p*<0.001
		LPS Vs LPS-Ex	*p*<0.01
		LPS Vs LPS-EE	*p*<0.05
		LPS Vs LPS-EE-Ex	*p*<0.01
60	F (4, 25) = 11.703;*p*<0.001	Control Vs LPS	*p*<0.001
		LPS Vs LPS-EE-Ex	*p*<0.01
80	F (4, 25) = 17.979;*p*<0.001	Control Vs LPS	*p*<0.001
		LPS Vs LPS-Ex	*p*<0.05
		LPS Vs LPS-EE	*p*<0.01
		LPS Vs LPS-EE-Ex	*p*<0.01
100	F (4, 25) = 16.344;*p*<0.001	Control Vs LPS	*p*<0.001
		LPS Vs LPS-Ex	*p*<0.01
		LPS Vs LPS EE and	*p*<0.01
		LPS Vs LPS-EE-Ex	*p*<0.001

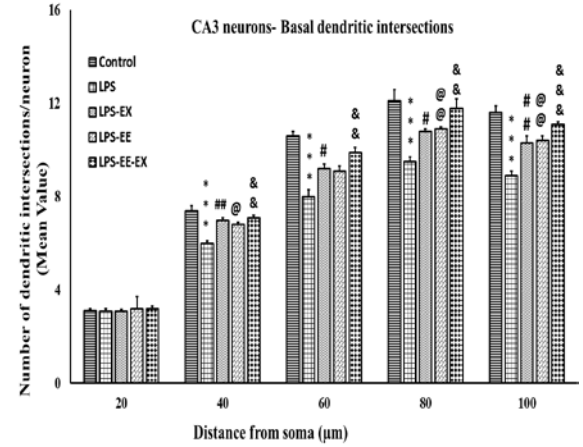

Figure 7. Effects of prenatal exposure to LPS and adolescent running exercise or being reared in a complex and enriched environment (EE) or a combination of the two, i.e., exercise and EE, on basal dendritic intersections of CA3 neurons. Symbols indicate levels of significance ****p*<0.001- LPS PND 60 Vs Control PND 60; && *p*<0.01 and &&& *p*<0.001- LPS PND 60 Vs LPS EE Ex PND 60; # *p*<0.05 and ## *p*<0.01 - LPS PND 60 Vs LPS Ex PND 60; @ *p*<0.05 and @@ *p*<0.01 LPS PND 60 Vs LPS EE PND 60. Each bar represents mean ± SEM.

4. Discussion

The results of the present study indicate that exposure to LPS induced prenatal inflammation resulted in decreased dendritic arborization of pyramidal neurons of CA3 region of the young adult hippocampus. This observations of the current study are in strong agreement with earlier reports; The prenatal LPS inflammation alters the dendritic arborization including significant reduction in the dendritic length in the medial pre-frontal cortex of PND 10 and PND 35, and in CA1 region at the age of PND sixty (Baharnoori *et al.*, 2009). Similarly, the maternal inflammation by LPS led to an altered thickness of CA1 region in young adult rats (Golan *et al*., 2005). LPS administration to pregnant rabbits during 28th day of gestation resulted in reduced dendritic arborization and spine density of thalamic neuron with decreased expression of synaptophysin in the newborn (Balakrishnan *et al.*, 2013). Although the researchers observed reduction in dendritic arborization of CA3 hippocampal neurons, the mechanisms underlying these morphological changes in the neurons are not addressed in the current study. However, evidence suggests that inflammatory cytokines, such as IL-6, IL-1β, and TNF- α, released in response to LPS exhibit deleterious effects on the neuronal development (Jeohn *et al*., 1998, Giovanoli *et al*., 2016). The inhibitory effect of TNF- α and IL-6 on the cortical and hippocampal dendritic arborization in *In vitro* neural culture cells (Gilmore *et al*., 2004, Neumann *et al*., 2002). The release of various neurotrophic factors such as brain-derived neurotrophic factor(BDNF) and nerve growth factor (NGF) in response to LPS induced prenatal inflammations affects the morphology of dendrites in cortex and hippocampus(Cohen-Cory *et al*., 2010, Ashdown *et al*., 2006). Thus, neuronal morphological alterations, or the reduced dendritic arborization of CA3 neurons, observed in the present study, may be attributed to the release of proinflammatory cytokines and altered levels of neurotrophins.

The cytoarchitecture of the neurons enables them to maintain their dynamic plasticity in response to various changes/stimulus of internal and external milieu. The ability of neurons to remodel its plasticity serves to protect against various adverse insults. The current results, also, demonstrated that the treadmill running exercise followed by housing in the complex environment during adolescent age significantly mitigates the prenatal LPS inflammation which causes morphological alterations of the dendrites. In the present study, it was noticed that the effects of treadmill exercise combined with environmental enrichment increases the dendritic arborization in the CA3 region to a greater extent, compared to effects of either the treadmill exercise only or environmental enrichment on its own.

The observations of this study are consistent with earlier studies; EE increased the brain weight of the mice that have been exposed to ethanol prenatally but not the cortical thickness (Wainwright *et al.*, 1993). The genes, downregulated by prenatal LPS exposure, that are specific for synaptic plasticity and transmission (such as EAAT2, BDNF, and TrkB) were upregulated by the enriched complex environment in early-life (Kentner *et al*., 2016). Evidence from clinical studies demonstrated the beneficial role of EE in autism (Woo *et al*., 2015), cerebral palsy

(Morgan *et al*., 2015), and schizotypical personality behavioral rehabilitation(Adrian Raine *et al.*, 2003).

A large body of growing evidence from various studies demonstrates that EE, and physical exercise improves the hippocampal neurogenesis (van Praag, 2008, van Praag *et al.*, 1999a, van Praag *et al.*, 1999b, Kempermann and Gage, 1999, Kempermann *et al*., 1997), counteracts the adverse effects of several prenatal insults like infection (Kentner *et al.*, 2016), stress (Morley-Fletcher *et al*., 2003, Lemaire *et al*., 2006), morphine(Ahmadalipour and Rashidy-Pour, 2015, Ahmadalipour *et al.*, 2015), and inhibits the progression of Alzheimer's disease (AD) - like pathology(Adlard *et al*., 2005). Also, EE, and physical exercise increase the level of nerve growth factors and neurotransmitter expression (Leggio *et al.*, 2005, Hüttenrauch *et al*., 2016, Rampon *et al*., 2000a), and improve dendritic arborization and the total length of dendrites of DG granule cells (Redila and Christie, 2006). An earlier report conducted by the researchers of this study, showed that the exposure to prenatal LPS inflammation resulted in cognitive deficits in young adult rats, whereas treadmill running exercise and being reared in a complex enriched environment attenuated the impaired memory and spatial abilities in Morris water maze (Thangarajan *et al*., 2015, Rajesh *et al*., 2016). These behavioral performances could be correlated to the neuronal morphological alterations observed in the current study.

5. Conclusion

The present study contributes to the existing literature by providing evidence that physical exercise combined with being reared in an enriched complex environment during early postnatal period can be an essential target for non-pharmacological interventions in preventing neuronal structural changes and cognitive deficits induced by prenatal LPS inflammations. However, further studies are still needed to examine and correlate the biochemical and molecular changes in response to prenatal exposure to LPS and the additive effects of treadmill exercise followed by enriched environment during the adloscent age.

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The Fumigant Toxicity of *Syzygium aromaticum* and *Cymbopogon citratus* Oils on Selected Life Stages of *Tribolium castaneum* (Coleoptera: Tenebrionidae)

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Abstract

In efforts to compliment research on biopesticides, attempts were made to determine the fumigant toxicity of essential oils from *Syzygium aromaticum* and *Cymbopogon citratus* against *Tribolium castaneum* larvae and adults under laboratory conditions. Four dosages (0, 50, 100 and 150µL/L) of each oil were applied in four replications. One-hundred larvae and unsexed adult populations of *T castaneum* were thus subjected to the oils in fumigant bioassays. α and β amylases activities in the fumigated life stages of *T. castaneum* were accessed. Toxicity data was subjected to One-way Analysis of Variance. Also, the relationship between the dosages of the *S. aromaticum* and *C. citratus* oils and the mortality rate among *T. castaneum* was investigated using regression analysis. Mortality rate was directly proportional to the dosages of both oils. The inhibitory potential of the oils, however, was only observed at the highest dose (150µl/L) for the adult stage, where low amylase activity was recorded. On the whole, amylase activity was higher in the insect stages fumigated with *S. aromaticum* oil. Similarly, significant positive linear correlation exists only between the mortality rate and the dosages of *S. aromaticum* oil ($r= 0.994$, $N = 4$, $P = 0.006$). The results gave cues on the action of the essential oils via some stress-related physiological activities in the insect stages that culminated in the killing of the insects.

Keywords: Fumigant toxicity, *Tribolium castaneum*, amylase activity, *Syzygium aromaticum*, *Cymbopogon citratus*, biopesticide.

1. Introduction

In the tropics, stored grains and grain products are significantly affected by pest insects (Jeyansakar *et al.*, 2016). An example of such pests is the red flour beetle, *Tribolium castaneum* (Herbst) (Coleoptera: Tenebrionidae), a polyphagous beetle commonly found in flour, flour products, cereal grains, grain legumes, and even spices. All forms of the insect life cycle can be found in infested stored commodities causing qualitative and quantitative food loss. In developing countries including Nigeria, post-harvest losses due to this insect-pest have been reported to be around 40 % (Pimentel, 1991).

In efforts to abate this occurrence, synthetic insecticides have been extensively and frequently used to control the red flour beetle. However, due to the growing concerns over the associated environmental and consumers' health, the effects on non-target organisms, insecticide resistance, and the high cost of application (Titli *et al.*, 2008; Ajayi *et al.*, 2014), alternative control measures are globally in focus nowadays (Arbes *et al.*, 2003). As such, there is a need to investigate substitutes that will achieve the desired level of efficacy, and more particularly, ones that are benign to the environment.

Already, the approaches of Integrated Pest Management give credence to this idea by adopting control tactics such as organic production to manage pests safely and effectively (Pedigo and Rice, 2009; Beatrice *et al.*, 2016). Similarly, the development of new pesticides from plants has been successfully leveraged on to reduce the pests' populations below the economic injury level (Adedire and Ajayi, 2003; Steven *et al*., 2017). Botanicals have been reported to contain defense compounds such as terpenes, alkaloids and enzyme inhibitors (Tinkeu *et al*., 2004; Schafer and Wink, 2009). These aproteic compounds exert effect on insect gut digestive enzymes, such as amylases (Octavio *et al*., 2002; Mazid *et al*., 2011).

The presence of an inherent rich source of bioactive chemicals make botanicals a promising source of potential alternatives to use as insect-control agents (Isman, 2008; Ballhorn *et al*., 2009; Mazid *et al*., 2011). Specifically, the essential oils of several spices such as the clove oil obtained from *Syzygium aromaticum* ((L.) Merril & Perry) have been found to possess fumigant toxicity to four major stored product pests, including *T. castaneum* (Shaaya *et al*., 1991; Merr and Perry, 2011). Souza *et al.* (2016) evaluated the toxicity of *Citrus aurantium* and *Cymbopogon citratus* on the grain weevil, and found out that the oils presented fumigating properties to be used in

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the management of the pests. Bachrouch *et al*. (2010) reported the potentiality of the control of one-seven-day-old adult *T. castaneum* by plant essential oils. In addition, these essential oils are extracted from botanicals that contain enzyme inhibitors and defending digestive enzymes as a natural defense response against pest attacks (Gerhenzon *et al*., 1991; Grayson, 1998). Tatun *et al*. (2014) reported that plant extracts exhibited inhibitory effects on the activity of amylase enzyme in *T. castaneum*. This characteristic qualifies botanicals as insecticides, as they are able to form complexes with insect digestive enzymes leading to poor nutrient utilization, growth retardation, and ultimately death (Fraga, 1988; Sami, 2014).

The current study investigates the acute fumigant toxicity of essential oils of Clove bud (*S. aromaticum*) and Lemon grass (*C. citratus* ((DC.) Staff) against adult and 4th instar larva of *T. castaneum* (Red flour beetle). In addition, the effect of these botanicals on α-amylase and β-amylase activities in these life stages was also explored.

2. Materials and Methods

2.1. Biological Materials

Adults of the American strain of *T. castaneum* used to start the experiments of this study were obtained from laboratory cultures of over one-hundred generations at the Department of Biology, of the Federal University of Technology in Akure Nigeria. The culture was raised on disinfested wheat flour at 30^{o}C ±2^{o}C and 75±5 % relative humidity. The White wheat flour was obtained from a grocery store in Akure metropolis, and was disinfested in Haier Thermocool freezer at 0^{o}F (-18^{o}C) for seventy-two hours. Newly emerged adults and fourth instar larva obtained from the insect culture were used for the bioassays. Oils of *S. aromaticum* clove bud (PCode 100198414), and lemon grass, *C. citratus* (PCode 101507375) were purchased from Sigma Aldrich Co. 3050 Spruce street St Louis, MO 63103 USA. The studies were conducted at the above-mentioned conditions in the laboratory at the Departments of Biology, and Biochemistry of the Federal University of Technology in Akure Nigeria.

2.2. Fumigation Bioassay

The fumigant toxicity bioassay was conducted on *T. castaneum* without flour to simulate surface treatment. This was done according to the methods of Negahban *et al*. (2007) with slight modifications. Air-tight plastic containers, of a one-litre size served as the fumigation chamber. One hundred (fifty pairs) adult insects were placed in each container. Whatman No. 1 (5mm Ɵ) filter paper was glued to the underside of the lid of the containers using a glue gun (AdTech HiTemp Project Pro; Made in Taiwan). Oils at 0, 50, 100 and 150µL/L were applied to the filter paper. The lid with the treatments was used to cover the insects immediately. The seam of each fumigation chamber was taped with Para film wrap to make it gas-proof. Four replicates were set for each dose of each treatment. The same procedure was done for the fourth instar larva. The control was similarly set-up without the application of essential oil. Mortality was observed five days post-treatment.

2.3. Preparation of Samples for Enzymatic Assay

The treated adults and larva were ground separately in a crucible mortar. A 0.5g of each ground insect of the two life stages was weighed into centrifuge tubes, and 500µL of 20mM sodium acetate buffer, pH 4.9 was added. The solution was homogenized at 10,000 rpm for twenty minutes at 4^{o}C. The supernatant was pipetted into Eppendorf tubes and stored in deep freezer at -20^{0}C until used.

2.4. Enzyme Assay

An aliquot of 0.5mL of the insect homogenates was pipetted into test tubes with a blank of 0.5mL reagent grade water in two replicates. The homogenates were incubated at 25^{o}C for three- four minutes to achieve temperature equilibration. Each tube received a 0.5mL starch solution at a fifteen-second interval. The mixture was then incubated at 25^{o}C for three minutes. At a fifteen second-interval, 1mL Dinitrosalicylic Acid (DNSA) color reagent was added to each of the test tubes, and the tubes were incubated in boiling water in a water bath for five minutes. After incubation, the tubes were allowed to cool at room temperature. 10 mL of reagent grade water was added to each tube and agitated. The content of each tube was poured into cuvette and inserted into a spectrophotometer. Absorbance was read at 540_{wv} (A_{540}). Enzyme activity was calculated as follows;

$$\text{Enzyme Activity} = \frac{A}{S \times T \times V}$$

Where; A= Absorbance of enzyme solution, S = Slope of standard graph, T = Time of incubation, V = volume of enzyme used.

2.5. Data Analysis

To determine acute toxicity, data obtained on the larvae and adults were converted to percentages and Arcsine transformed. The transformed data were subjected to one-way Analysis of Variance (ANOVA). Where significant difference exists, New Duncan's Multiple Range Test (NDMRT) was used to separate the means. The data obtained from biochemical analysis was presented as a mean of two replicates. Regression analysis was used to investigate the relationship between the dosages of the oils and the mortality rate among the *T. castaneum*. All analyses were conducted using the Microsoft excel (2016) and Statistical Package for Social Sciences (SPSS) version 21.

3. Results

3.1. Fumigant Toxicity of S. aromaticum and C. citratus on Larva and Adult T. castaneum

Mortality of larva was dose-dependent (Figure 1). There was no death of larvae in control and fumigation done with 50µL/L of the *S. aromaticum* oil. *C. citratus* was more toxic to larvae than *S. aromaticum*. The highest dose of *S. aromaticum* and *C. citratus* oils (150µL/L) elicited the greatest mortality, 30 % and 41 % of the larvae respectively. (Figure 1). Lower mortality of the beetles was recorded at 100µL/L for *S. aromaticum* (27.5 %) and *C. citratus* (28.5 %) (Figure 2).

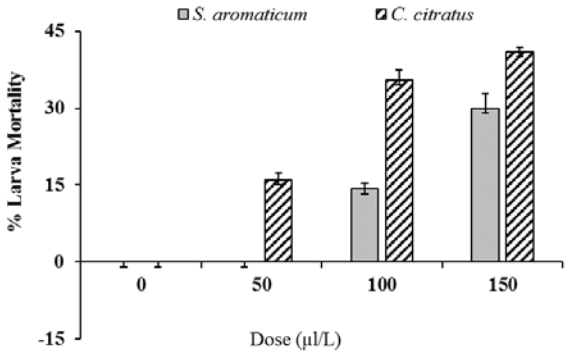

Figure 1 . Percentage mortality of *T. castaneum* larvae fumigated with essential oils of *Syzigium aromaticum*, and *Cymbopogon citratus* (n = 4)

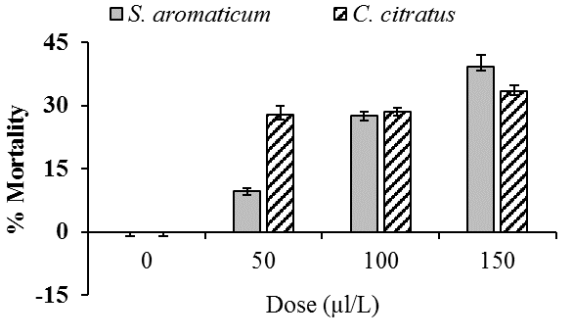

Figure 2. Percentage mortality of adult *T. castaneum* fumigated with essential oils of *Syzigium aromaticum*, and *Cymbopogon citratus* (n = 4)

3.2. Activities of α and β amylase in T. castaneum Larvae and Adult Fumigated with Essential Oils of S. aromaticum and C. citratus

The enzyme activities increased with the increasing of the oil dosage (Figures 3). However, the highest α amylase (50 mol/min/mL) and β amylase (13.64mol/min/mL) activity in the *C. citratus*-fumigated adults, was recorded in the insects fumigated with 100 µL/L (Figure 3 A, B). The α and β - amylase activities were higher in the insect stages fumigated with *S. aromaticum* oils except for β - amylase in larvae at 150 µl/L (Figure 3 C, D).

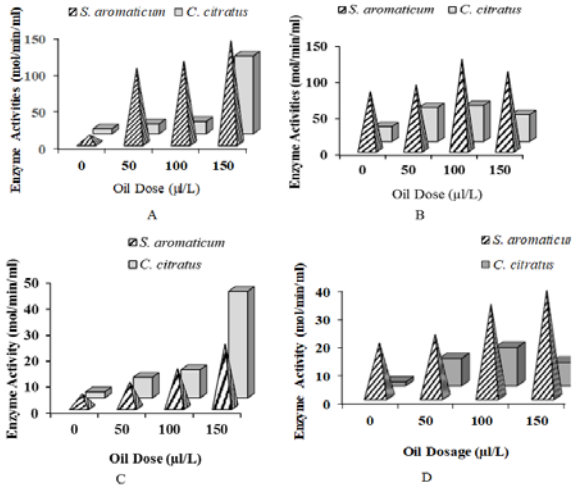

Figure 3. α-amylase ([A] larvae and [B] adult) and β-amylase ([C] larvae and [D] adult) activities in *T. castaneum* fumigated with essential oils of *Syzygium aromaticum*, and *Cymbopogon citratus.*

3.3. Relationship between Dosages of S. aromaticum and C. citratus Oils and Mortality Rate in T. castaneum Larvae and Adults

The relationship between the dosages of the *S. aromaticum* and *C. citratus* oil and the mortality rate among the *T. castaneum* larvae and adults is presented in Table 1. Regressing mortality rate in the flour beetle larvae against increasing concentration levels of the *S. aromaticum* oil (r = 0.941, N = 4, *P* = 0.059) and *C. citratus* oil (r = 0.980, N = 4) showed a positive correlation, the correlation was found to be significant (*P* = 0.020) for larvae treated with *C. citratus* oil. Similarly, in the adults, there was a significant positive linear correlation between the mortality rate among the insects and the dosages of *S. aromaticum* oil (r = 0.994, N = 4, *P* = 0.006) and *C. citratus* oil (r = 0.861, N = 4). The correlations were, however, found to be insignificant (*P* = 0.139) for the insect adults treated with *C. citratus* oil.

Table 1. Relationship between dosages of the *S. aromaticum and C. citratus* oils and the mortality rate of the *T. castaneum* larvae and adults.

Essential oil	Correlation coefficient (r)	Regression equation
S. aromaticum (larvae)	0.941	Y = -4.565 + 0.209X
C. citratus (larvae)	0.980	Y = 1.750 + 0.285X
S. aromaticum (adult)	0.994	Y = -1.200 + 0.271X
C. citratus (adult)	0.861	Y = 7.250 + 0.203X

4. Discussion

All doses of the essential oils assayed elicited some level of toxicity to *T. castaneum* larvae and adults more than in the controls. This confirms the presence of bioactive compounds in the oils of these plants (El hag *et al*., 1999; Samarasekera *et al*., 2006; Chaieb *et* al., 2007). The observed low level of toxicity to the life stages of the beetles does not undermine the efficacy of the oils, rather, it suggests that the essential oils may not be an effective fumigant against *T. castaneum* at low doses. Therefore, it may be necessary to increase the dosage to achieve a stronger degree of toxicity. This finding is in agreement with earlier findings on the effects of *C. citratus* oil on several insect pests. The essential oils of *C. citratus* and *S. aromaticum* had been reported toxic to *T. castaneum* in a dose-dependent manner (Verbel *et al*., 2010; Rafeeq *et al*., 2016).

The oil of *C. citratus* was more toxic to *T. castaneum* than that of *S. aromaticum.* The monoterpene hydrocarbons which are characterized by geranial, neral, and myrecene and accounts for about 95 % of the total oil in *C. citratus* have been demonstrated to have high activity against insect pests (Lee *et al*., 2008; Josphat *et al*., 2011). In addition, Mondal and Khalequazzama (2006) investigated contact and fumigant activity of three essential oils, namely *Elettaria cardamomum, Ctenium aromaticum* and *S. aromaticum* against *T. castaneum* larvae and adults and reported that *S. aromaticum* was the least effective as a fumigant.

Starch is a major component of food grains among cellulose and xylose. A number of insects rely on cellulases and amylases for utilizing alpha and beta

polymer of glucose, as a source of carbohydrates. Owing to its exclusive dependency on starch, *T. castaneum* produces a raft of starch-hydrolyzing enzymes such as α and β amylase to help hydrolyze carbohydrates (Chen *et al*., 1992; Sami, 2014). Because the population of *T. castaneum* used in this study were raised on wheat flour, another source of starch, before being fumigated, it is probable that active carbohydrate metabolism had already begun in the insect before being exposed, hence the probable rational for the observed low mortality of insects in this study. The insects' restlessness and erratic movement during fumigation indicate that the oils induced stress in the insects. Increased enzymatic activities in this insects are a physiological response to adapt with abnormal condition.

Upon exposure to toxic substances, changes in enzymatic reactions have been reported to be generally developmental and stage-specific in a number of insects including *Tribolium spp* (Mediola-Olaya *et al*., 2000; Bandani and Balvasi, 2006). In these species, a higher enzyme activity is an occurrence commonly recorded in the adults more than in the larvae. Accordingly, the findings in this research, which reveal higher levels of enzyme activity in the adult *T. castanuem* more than in the larvae, appear to be well correlated with the reported findings.

The reduction in α- amylase activity of the adult beetles at the highest dosage of the oils may be precipitated by the defense compounds including inhibitors of digestive enzymes which act on insect enzymes (Franco *et al*., 2002; Jing *et al*., 2005). It is probable that these defense compounds are in proportions high enough to exert effect compared to other levels of dosages administered to *T. castaneum*. More so, since the 150 µL/L oil exhibited a higher proportion of toxicity to the insects relative to the other doses tested, it is most likely that this dose contains high enough amounts of inhibitory compounds that propagated the decline recorded in amylase activity. Moreover, the cytotoxic effects of the essential oils on the epithelial cells of the midgut of *T. castaneum* have been implicated in the reduced α- amylase activity (Tatun *et al*., 2014). While no cytological study was done on *S. aromaticum* and *C. citratus* oils, it could, however, be a probable cause in the decline in amylase activity recorded.

This study shows that the *T. castaneum* larvae at the varied level of doses used had higher enzyme activity than the adults. The chitinous exoskeleton found in the adults compared to the larvae reflects a probable scientific rational for the variation in the mortality rates as this feature may account for the reduction in the quantity of the oil that eventually makes it into the insect body.

5. Conclusion

While validating the potentiality of essential oils in the control of storage pests, it is important to investigate their variety and understand how they exert their effects. The results show that the oil of *C. citratus* was more toxic to *T. castaneum* than to the *S. aromaticum* oil. Generally, insect life stages fumigated with *S. aromaticum* had higher enzyme activity compared to those exposed to *C. citratus*. However, given the generally observed low level of toxicity, there is a need for further research on the essential oils and enzyme activity. The enzyme activity in particular, elicited by exposure to plant oils, needs to be further characterized by gene sequencing, if the industrial application of bio-pesticides is to be fully explored. This sentiment is expressed because, at the corresponding highest toxicity dose, both oils led to a decline in the amylase activity.

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Single Nucleotide Polymorphisms in *TLR4* Gene and Endometritis Resistance in River Buffalo *(Bubalus bubalis)*

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Abstract

Toll-like receptor (TLR) genes play a crucial role in pathogen recognition and subsequent activation of the innate immune response. The role of *TLR4*in the initiation of inflammatory and immune response makes it a suitable candidate gene for marker-assisted selection to enhance disease resistance in dairy animals. The present study was undertaken to characterize the distribution of single nucleotide polymorphisms (SNPs) in *TLR4* coding region, and to test their role as potential risk factor for the occurrence of endometritis in river buffalo Egyptian breed (*Bubalus bubalis*). The analysis of the entire coding sequences (CDs) enabled the identification of 13 non-synonymous SNP. A statistical correlation was observed between resistance to endometritis and the genotype CC at the 2464 C >A locus and AA genotype at the 2465 A > C. Moreover, a correlation was seen between susceptibility to endometritis and the heterozygous and recessive homozygous genotypes of these SNPs. Haplotype reconstruction of the *TLR4* gene revealed a statistical significance of haplotype frequencies between healthy and diseased cases by performing a permutation test which retrieved significant association with the occurrence of the disease in the coding region of *TLR4*.PolyPhen 2 analysis revealed two amino acid substitutions which may have a potential structural and functional significance. The current study describes some novel SNPs in *TLR4* gene of river buffalo Egyptian breed and their association with the endometritis disease, therefore suggesting a possible positional marker in the buffalo genome that may be related to such a disease.

Keywords: *Bubalus bubalis*, *TLR4*, Endometritis, SNPs , Genotype .

1. Introduction

Water buffalo plays a pivotal role in the livestock and agricultural economy of many developing countries including Egypt. The current world water buffalo population is estimated at 195 million heads including both river and swamp buffaloes. Egyptian buffaloes are of the river type with a population of 4 million heads, it ranks third after the Indian (over 115 millions) and Pakistani buffalo (31.7 millions) (Lewandowski 2015).

Although there are many advantages for raising water buffalo, breeders and farmers have been facing many problems such as poor reproductive efficiency and high incidence of infertility (Michelizzi *et al*., 2010). Endometritis is the most common uterine disease observed in buffaloes slaughtered at abattoirs, and is one of the main causes of infertility in both cattle and buffalo (Ajevar *et al*.,2014; Azawi *et al*., 2008). The severity of endometritis is dependent in part on the type of bacteria present; moreover, the establishment and persistence of the uterine infection are also influenced by the presence of a suitable uterine environment, genetic factors, and the animal's innate and acquired immunity (Williams *et al*.,2005).

Mammalian Toll-like receptors (TLRs) are a key family of innate immune proteins and a major class of pattern-recognition receptors (PRRs) (Medzhitov 2001). TLRs recognize broad classes of pathogen- associated molecular patterns (PAMPs), and play an essential role in initiating and directing immune responses to pathogens (Takeda *et al*., 2003 ; Takeda and Akira, 2005).

Single nucleotide polymorphisms (SNPs) can occur anywhere in the genome. Leveque *et al*., (2003) reported that polymorphisms within TLR genes are associated with variations in disease resistance traits in livestock.

The *TLR4* gene in water buffalo consists of three exons and two introns, and is located on chromosome number 8 (Gulhane and Sangwan, 2012). *TLR4* is a member of the TLR family which is highly expressed in the endometrial cells of the uterus, and play a key link between endometritis and immune system response; therefore, they can be considered as detection markers for the endometritis disease (Ganesan *et al*., 2013;Yeo *et al*., 2013 ; Ju *et al*., 2014). Polymorphisms of *TLR4* exons in buffalo

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lead to the alteration of resistance and susceptibility for the disease occurrence, and make it a suitable candidate gene for use in marker-assisted selection (Mitra *et al*., 2012).

The aim of this study is to identify SNPs in the full CDs of *TLR4* gene performing a case-control study to test its role as potential risk factor for the endometritis occurrence in river buffalo Egyptian breed, with the purpose of enhancing available genetic tools for the improvement of health and production of an animal considered of increasing worldwide economic importance. Additionally, the current study characterizes *TLR4* protein architecture domains, where TIR domain plays an important role in inflammatory response activation since the number and structure of LRRs of TLRs have a significant effect on the function of pathogen recognition system among species.

2. Materials and Methods

2.1. Study Design

Genetic association between *TLR4* gene SNPs and endometritis disease was studied by a case-control approach on uterine tissues which were collected from forty Egyptian buffaloes (river buffalo) at the slaughter house. The forty samples were divided equally to apparently healthy uteri and clinically infected ones according to the physical examination and presence of abnormal secretions and inflammation signs in uterine tissues (Williams *et al.,* 2005; Sheldon *et al.,* 2009). Bacteriological processing was performed on all forty uterine samples to confirm the samples identification detected by visual inspection, and to identify bacterial pathogens from clinically diseased samples (Collee *et al*., 1996; Quinn *et al.,* 2011).

2.2. TLR4 Sequencing

Total genomic DNA was extracted from uterine samples using DNeasy Blood and Tissue kit (Qiagen, USA) according to the manufacturer's instructions. Five specific PCR primers were designed to amplify entire CDs of three exons of *TLR4* gene using published *Bubalus bubalisTLR4* gene (accession no. JN786600) and the web interface primer3 as shown in Table 1. The *TLR4* coding sequence was amplified using five primers according to the following thermal profile: initial denaturation at 95°C for 1-3 minutes, followed by forty cycles of thirty seconds at 95°C, thirty seconds at ™ann(C) which is the annealing temperature specific for each primer (Table 1) and one minute at 72°C, with a final extension for one minute. PCR reactions were performed with PCR Master Mix kit (Thermo Fisher Scientific, UK), which included in addition, 50ng of genomic DNA and 10μM of each primer (forward and reverse) in a final volume 50 μL. Amplicons were purified according to the manufacturer's instructions (QIAquick PCR Purification Kit, Qiagen) and were bi-directionally sequenced by Laragen company (USA).

Table 1. PCR primers, annealing temperatures and amplicon size of amplified *TLR4* exons.

Amplicon (bp)	PCR ™ann(C)	Sequence (5`-3`)	CDs of *TLR4* gene
785	53°C	F: GACAGCCATCTATAAGCCAAGG	TLR4 A (CDs_Exon1)
		R: TGTCTGTTTGCAAATGAACCT	
273	56°C	F: AGAGTTGCTGGGAAGTCTGC	TLR4 B
		R: AACATTCCTCCTTGTACAGTGGT	(CDs_Exon2)
985	55°C	F: GCATTGTTATATCTGTGTGGAGACC	TLR4 C
		R:TGAGATCTAGATACTGAAGGCTTGG	(CDs_Exon3)
953	54°C	F: CGAATTCTCAGGGGACGATA	TLR4 D
		R: GCTCTGCACACATCATTTGC	(CDs_Exon3)
953	55°C	F: GACTGCAGTTTCAACCGTATCA	TLR4 E
		R:TGGCAGCATTTACTTGTTAACTGA	(CDs_Exon3)

2.3. SNP Selection and Genotyping

Sequences were analyzed by multiple alignment using CLUSTAL–W program (Thompson *et al.,* 1994). The forty river buffalo Egyptian breed sequences of the *TLR4* gene were aligned with CDs of publicly available GenBank: JN 786600. Each forward and reverse sequence from a single DNA sample was aligned against each other to generate a consensus sequence for each sample and to identify polymorphisms among the samples, in addition to the confirmation of polymorphic sites by visual examination of sequence's charts.

2.4. Genetic Association and Protein Analysis

Genotype frequencies were tested for deviation from Hardy-Weinberg equilibrium (HWE) by using Fisher's Exact test, where the allelic and genotypic frequencies were carried out for each SNP independently.

The univariate logistic regression model was performed for the SNPs that were significantly associated with the disease. Odds Ratio test (OR) was calculated with 95 % confidence interval (CI) (McHugh, 2009). All statistical analyses were performed using R statistical program, and *P*-value was corrected using Bonferroni method (Bland and Altman, 1995). Haplotype reconstruction was performed using PHASE software, (version 2.1) (Stephens *et al*., 2001). The potential impact on protein structure and function due to amino acid substitutions was performed by PolyPhen-2 software (Polymorphism Phenotyping V2) with default parameters (Adzhubei *et al*., 2010), and the colored figures are available at http://genetics.bwh.harvard.edu/cgi-bin/ggi/ggi2.cgi. Protein domain has been predicted from the CD sequence by using the Conserved Domain Database available at (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) (Aron *et al*., 2009).

3. Results and Discussion

3.1. Bacteriological Analysis

In a previous study, Osman *et al.* (2015) aimed to characterize the transcripts of TLRs: TLR1, TLR2, TLR4, TLR6 and TLR10 in *Bubalus bubalis* with endometritis. Quantitative real-time (q-PCR) assays were performed to detect transcript expression profiles of these TLRs in liver, mammary gland, ovary and uterus of *Bubalus bubalis* with and without endometritis. The results showed that the

transcript profiles of TLRs especially TLR2 and TLR4 were significantly different in mammary glands, ovary and the uterus.

The current study focused on an important *TLR* gene, *TLR4*, which has the ability to recognize endotoxins associated with Gram-negative bacterial infections (White *et al.,* 2003 ; Davies *et al.,*2008; Mariotti *et al*., 2009 ; Mitra *et al.,* 2012 ; Alfano *et al.,* 2014). As shown in Table 2, *Escherichia coli* was the highly isolated bacterium from the diseased samples.

Table 2. Identification of bacteria, isolated by aerobic and anaerobic culture of uterine swabs, according to their expected pathogenic potential in the uterus.

1	2	3
E. coli (45.5)	*S. aureus* (31.58)	*Micrococcus spp.* (23.68)
	S. pyogenes (26.32)	*Klebsiella spp.* (33.3)
		S. epidermidis (18.42)
		Proteus spp. (21.2)

-Categories are: (1) recognized uterine pathogens associated with uterine endometrial lesions; (2) potential pathogens frequently isolated from the bovine uterine lumen and cases of endometritis but not commonly associated with uterine lesions; (3) opportunist contaminants transiently isolated from the uterine lumen and not associated with endometritis. Numbers in parenthesis indicate the percentage of a total of 20 samples isolates.

3.2. SNPs Identification and Protein Analysis

The analysis of the entire CDs of *TLR4*, which was deduced from five overlapped amplified fragments, enabled us to identify thirteen polymorphic sites. All SNPs were bi-allelic and non-synonymous with eight nSNPs being transversions and five nSNPs being transitions. In addition, two dinucleotide SNPs in the CDs of exon 3 (576/577 TG > GA & 2464/2465 AC > CA) were identified. The *TLR4* CD sequences generated from healthy and diseased cases were submitted to GenBank with the accession numbers KU984440 and KU984441, respectively. For protein analysis, the majority of amino acid substitutions were detected to be benign. Only two substitutions (I 271 R and F272 L), which were found within *TLR4* coding region, were observed to be possibly damaging, and may have a potential impact on the alteration of protein structure and function (Adzhubei *et al.,* 2013). It is to be noted that the change of polarity from nonpolar Isoleucine to polar Arginine (I 271 R) might also affect the protein structure and function (Allen *et al*., 2013). This effect of change in protein function, related to that of amino acid polarity of *TLR4* SNPs, was also reported in cattle by White *et al.* (2003). It was also previously reported that non-synonymous SNPs that modify amino acids polarity in TLRs may affect ligand binding and recognition (Zhang e*t al.,* 2014). Furthermore, amino acid substitutions that alter the amino acid polarity may also have a potential impact on host immune responses and resistance to diseases (Shinkai *et al*., 2006).

Table 3 shows all the SNPs, their positions in TLR4 CDs, the protein domain and PolyPhen-2 analysis of amino acid substitution effects of these SNPs. Five non-synonymous SNPs were detected in Leucine-rich repeats (LRR) domains of *TLR4*, which might have a role in altering its ability to identify extracellular pathogens (Fujita *et al*., 2003 ; Seabury *et al.,* 2007). Furthermore, polymorphisms that occur in LRR domains may cause changes in responsiveness towards pathogenic micro-organisms (Matsushima *et al.,* 2007).

3.3. Case-Control Study Analysis

As a first step for the genetic association study, Fisher`s Exact test was carried out for the calculation of allele and genotype frequencies for each SNP (McDonald, 2009), where it was found that all *TLR4* SNPs conformed to Hardy-Weinberg equilibrium (P >0.05) (Salanti *et al*., 2005). Only two SNPs (2464 M (C/A) & 2465 M (A/C)) in the coding region were found to be statistically significant (P<0.01) in the distribution of allele and genotype frequencies between healthy and diseased cases (Table 4).

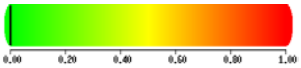
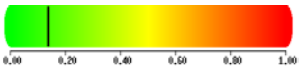
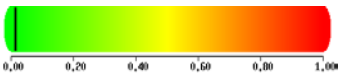
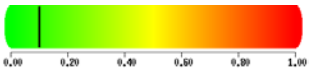
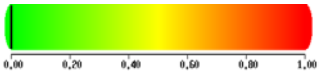
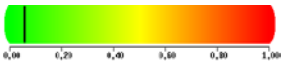
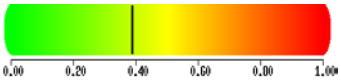
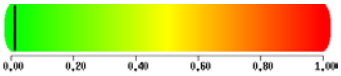
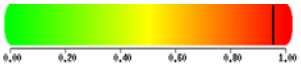
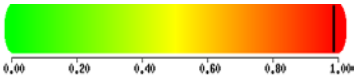
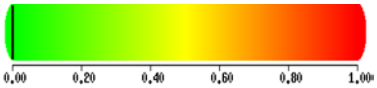
3.4. Odd Ratio Test Analysis for Statistically Significant SNPs

The two statistically significant SNPs [2464 (C/A) and 2465 (A/C)], associated with endometritis disease (P<0.01) (Table 4) were subjected to further analysis. The logistic regression model was performed to calculate the Odd Ratio (OR) for each SNP.

The OR test analysis revealed the presence of associations between the two statistically significant SNPs [2464 (C/A) and 2465 (A/C)] and resistance/susceptibility to the endometritis disease. Specifically, a correlation was observed between resistance to the endometritis disease and the genotype CC at the 2464 C >A locus and AA genotype at the 2465 A > C locus [P=0.006, OR=0.2411 and 95% Cl=0.134-0.436]. Moreover, a correlation was detected between susceptibility to endometritis and the heterozygous and recessive homozygous genotypes of those SNPs (OR: 2.125 and 6.00; P: 0.01 and 0.002, respectively; Table 5). Despite the relatively small number of samples used, the results give a rather good idea about the association between TLR4 genotypes and resistance/ susceptibility to endometritis. Those results are in good agreement with: Cargill and Womack, 2007 ;Uenishi and Shinkai, 2009 ; Jann *et al.,* 2009 ; Koets *et al.,* 2010 ; Plantinga *et al.,* 2012 ; Tschirren *et al.,*2013 ; Alfano *et al.,* 2014, who reported that TLRs polymorphisms are associated with resistance and susceptibility to diseases in many species.

Previous studies have demonstrated the influence of polymorphisms on susceptibility to several bacterial diseases, where *TLR4* polymorphisms were found to increase the risk of infections like tuberculosis (Zhang *et al.,* 2013 ; Sun *et al.,* 2015), urinary tract infections (Hawn *et al.,*2009), Mastitis (Kannaki *et al.,* 2011; Sharma *et al.,* 2006), and other disease conditions (Mucha *et al.,* 2009 ; Schnetzke *et al.,*2015). The relation between endometritis occurrence and genetic polymorphism of TLRs in humans and cattle was previously reported (Taylor *et al.,* 2012 ; Pinedo *et al.,* 2013). In this respect, several polymorphisms have been found in the bovine *TLR4* gene, 12 SNPs affecting ligand binding domain (White *et al*., 2003), and four SNPs were associated with susceptibility to Map infection (Mucha *et al*., 2009).

Table 3. Detected SNPs and their effect on protein function in *TLR4* CDs.

CDs_Exon	SNPs positions	Amino acid substitution	Protein domain	Amino acid substitution effect PolyPhen-2 software (Polymorphism Phenotyping V2) [Adzhubei *et al*. 2010]
CDs_Exon1	44 Y(C>T)	Thr (Polar) 15 Met (Non Polar)	-*	This mutation is predicted to be BENIGN with a score of 0.000 (sensitivity: 1.00; specificity: 0.00) 
CDs_Exon 1	66 W(A>T)	Arg (Polar) 22Ser (Polar)	LRR_R1	This mutation is predicted to be BENIGN with a score of 0.137 (sensitivity: 0.92; specificity: 0.86) 
CDs_Exon 3	572 M (C>A)	Tyr (Polar) 191Ser (Polar)	LRR_8	This mutation is predicted to be BENIGN with a score of 0.013 (sensitivity: 0.96; specificity: 0.78) 
CDs_Exon 3	576 K (T>G)	His (Polar) 192Gln (Polar)	LRR_8	This mutation is predicted to be BENIGN with a score of 0.101 (sensitivity: 0.93; specificity: 0.85) 
CDs_Exon 3	577/579 R(GA>AG)	Lys (Polar) 193 Glu (Polar)	LRR_8	This mutation is predicted to be BENIGN with a score of 0.001 (sensitivity: 0.99; specificity: 0.15) 
CDs_Exon 3	647 R(G>A)	Gly(Non Polar) 216 Asp (Polar)	LRR_R1	This mutation is predicted to be BENIGN with a score of 0.055 (sensitivity: 0.94; specificity: 0.84) 
CDs_Exon 3	662 R(G>A)	Gly(Non Polar) 221 Asp (Polar)	-*	This mutation is predicted to be BENIGN with a score of 0.389 (sensitivity: 0.90; specificity: 0.89) 
CDs_Exon 3	672 M(A>C)	Lys (Polar) 224 Asn (Polar)	-*	This mutation is predicted to be BENIGN with a score of 0.011 (sensitivity: 0.96; specificity: 0.78) 
CDs_Exon 3	812 K(T>G)	IIe (Non Polar) 271Arg (Polar)	-*	This mutation is predicted to be POSSIBLY DAMAGING with a score of 0.964 (sensitivity: 0.76; specificity: 0.94) 
CDs_Exon 3	816 M(C>A)	Phe (Non Polar) 272Leu (Non Polar)	-*	This mutation is predicted to be PROBABLY DAMAGING with a score of 0.985 (sensitivity: 0.74; specificity: 0.96) 
CDs_Exon 3	2464/2465 M (AC>CA)	Gln (Polar) 822Thr (Polar)	-*	This mutation is predicted to be BENIGN with a score of 0.001 (sensitivity: 0.99; specificity: 0.15) 

-SNPs positions were calculated by taking the ATG start codon as position 1 on the GenBank sequence: JN786600

- Amino acids substitutions are considered according to the ATG start codon

-* Protein position without known function

Table 4. Corrected *P*-values for allelic and genotypic frequencies for each SNP of *TLR4* CDS.

SNPs position	Corrected P value of allelic frequency	Corrected P value of genotypic frequency
44 Y(C/T)	0.05	0.14
66 W(A/T)	0.05	0.14
572 M(C/A)	0.97	0.99
576 K(T/G)	0.96	0.98
577 R(A/G)	0.97	0.99
579 R(G/A)	0.97	0.99
647 R(G/A)	0.96	0.98
662 R(G/A)	0.96	0.98
672 M(A/C)	0.96	0.98
812 K(T/G)	0.96	0.98
816 M(C/A)	0.96	0.98
2464 M(C/A)	0.004*	0.0012*
2465 M(A/C)	0.004*	0.0012*

-*Statistically significant values (P <0.01). Fisher Exact test was used because of the small number of samples. Bonferroni was used for p value correction.

Table 5. Polymorphic sites including genotypes of statistically significant differences in frequency distribution between healthy and diseased cases.

SNPs	Genotype	*P*-value	OR	95% Cl
CD 2464	CC	0.006*	0.2411	0.134-0.436
M(C/A)	CA	0.01*	2.125	1.1962-3.7750
	AA	0.002*	6.000	1.969-18.275
CD 2465	AA	0.006*	0.2411	0.134-0.436
M(A/C)	CA	0.01*	2.125	1.1962-3.7750
	CC	0.002*	6.000	1.969-18.275

-*Statistically significant p values (p <0.05) and ORs. CL: confidence level.

3.5. Haplotype Prediction

The determination of haplotype reconstruction for several SNPs in one gene is considered to be a powerful tool to provide more information about genotype-phenotype associations than individual SNPs (Ciampolini *et al.*, 2007). Haplotype reconstruction based on *TLR4* polymorphisms performed by PHASE software, generated 21 possible haplotypes. The most frequent haplotype within healthy cases was TTCTAGGGATCCA which displayed 30 % frequency, while CACTAGGGATCAC was the most frequent haplotype within diseased cases with a 31 % frequency. The software PHASE revealed the presence of a statistical significance (P value=0.02) of haplotype frequencies between healthy and diseased cases, which indicated significant association between haplotype frequencies and the occurrence of endometritis disease in the coding region of *TLR4*.

Haplotype reconstruction of TLRs and its association with the disease occurrence have been reported in humans (Ferwerda *et al*., 2007 ; Bochud *et al*., 2008) ,cattle (Jann *et al*.,2009) and buffaloes (Alfano *et al*., 2014).

4. Conclusion

Polymorphisms and mutations of TLRs interfere with the innate immune activation due to the reduction of the protein ability to recognize pathogen associated molecular patterns (PAMPs). The current study is focused on an important Toll-Like receptor gene, *TLR4*, which has the ability to recognize endotoxins associated with gram-negative bacterial infections. It suggests the presence of novel polymorphic sites in *TLR4* gene in the river buffalo Egyptian breed and their association with the endometritis disease occurrence. Studies of the genetic factors involved in complex diseases may help identify their underlying physio-pathological pathways, which will improve our ability to understand the disease in its entirety, and to determine the risk of developing it. This will pave the way to the improvement of disease resistance in herds by selective breeding, and also to the identification and synthesis of innovative drugs.

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Spermatogenesis and Steroidogenesis Functions of Rat Testis Following Exposure to *Alafia barteri* Leaf Extracts

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Abstract

In Africa and other continents, herbal remedies have played essential roles in the treatment of all kinds of diseases since early times. This study is focused on the impact of *Alafia barteri* (*A. barteri***)** leaf extract on spermatogenesis and steroidogenesis in rats. Twenty adult male wistar rats randomly were chosen and assigned into four groups of five (n=5) rats each, consisting of a control which received only 2 mL/kg of normal saline and the treatment groups receiving the doses of 100, 300 and 500 mg/kg of body weight daily for twenty-eight days via gastric gavage. The parameters tested included sperm quality, reproductive hormones, superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX), and malondialdehyde (MDA) levels. Sperm quality, total serum testosterone, SOD, CAT, and GPX levels were significantly increased in the *A. barteri* groups in comparison to controls ($P < 0.05$). Also, rats in the *A. barteri* groups showed a significant decrease in the level of plasma MDA ($P < 0.05$) in comparison to controls. There was a decrease in Follicle Stimulating Hormone (FSH), but no significant increase in the levels of luteinizing Hormone (LH). The administration of *A. barteri* extract significantly increased the sperm quality and improved the profertility properties; these profertility properties can be exploited in male fertility therapy.

Keywords: *Alafia barteri*, Catalas, Spermatogenesis, Superoxide Dismutase, Testosterone, Steroidogenesis

1. Introduction

Infertility is one of the major health challenges in life. Approximately 30 % of infertility can be attributed to male factors (Carlsen *et al*., 1992; Isidori *et al*., 2006). Several conditions can interfere with spermatogenesis and reduce sperm production and quality (Arash *et al.*, 2009). Among these factors include drug treatment, chemotherapy, toxins, air pollution, and insufficient vitamins' intake which may also have harmful effects on spermatogenesis and the production of normal sperm (Mosher and Pratt, 1991). Several diseases, such as coronary heart diseases, diabetes mellitus, and chronic liver diseases may (also) interfere with the spermatogenesis process, and therefore sperm quality and quantity may be altered by these diseases (Shalaby and Mouneir, 2010).

Antioxidants are significant agents, which can contribute to the overall health of the organism (Saliha *et al.,* 2014). For example, Polyphenols are dietary antioxidants associated with redox activities and have quite beneficial effects on health (Scalbert *et al.,* 2005). Oxidative stress is a critical process that can (be) involved in multiple conditions such as infertility and inflammation (Dragsted, 2003). However, infertility and inflammation can be controlled with antioxidant supplements (Scalbert *et al.,* 2005). Antioxidant enzymes, such as catalase (CAT), superoxide dismutase (SOD) and glutathione (GSH), have a significant role in establishing a balance between reactive oxygen species (ROS) and antioxidant levels in serum (Khaki *et al.*,2009). Therefore, the use of antioxidants may improve spermatozoa viability and longevity (Khaki *et al.*, 2009).

Alafia barteri is a high-climbing, scandent shrub with small, pure white or pink flowers (Irvine, 1961). It is used in ethnomedicine for the treatment of sickle cell anaemia, rheumatism, eye infections, febrifuges, toothache, in addition to being used as chew sticks (Adekunle and Okoli, 2002). The twining stem of *A. barteri* is used for the treatment of fever, inflammation, and the roots are used in the manufacture of binding materials (Burkill, 1985; Daziel, 1937; Irvine, 1961; Nadkarni, 1976; Leeuwenberg, 1997). Antifungal properties of ethanol and water extracts of *A. barteri* leaves were reported (Adekunle and Okoli, 2002). In South-Western Nigeria (Lagos), *A. barteri* has been used for the treatment of malaria (Olowokudejo *et al.,* 2008). The decoction of the root and leaves of this plant can be taken internally or applied externally to treat rheumatic pain, toothache, and eye infections (Odugbemi, 2008). A preliminary phytochemical report on the stem extracts of *A. barteri* showed the capability of reducing sugars, and the presence of steroids, flavonoids and anthraquinones (Hamid and Aiyelaagbe, 2011). To the best of our knowledge, the spermatogenesis and

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steroidogenesis functions of *A. barteri* have not been established. This study is aimed at investigating the impact of *Alafia barteri* leaf extract on spermatogenesis and steroidogenesis in rats.

2. Materials and Methods

2.1. Plant Material

The leaves of *A.barteri*, were collected from Ipale forest, Irawo (7°25' N, 3°31'S), Oyo state Nigeria, in May, 2017. The plant samples were authenticated by Prof. Ogunkunle of the Department of Pure and Applied Biology at Ladoke Akintola University of Technology, Ogbomoso in Nigeria and a voucher a specimen was deposited at the same Department for reference purposes.

2.2. Preparation of the Plant Extract

The leaves were thoroughly washed in sterile water, and were air dried to a constant weight in the laboratory. The air-dried leaves were weighed using Gallenkamp (FA2406B, England) electronic weighing balance, and were milled with automatic electrical Blender (model FS-323, China) to obtain a powdered form. The powdered plant sample (420 g) was extracted with 96 % ethanol for twenty-four hours, at room temperature with constant stirring. This process was repeated twice for a complete extraction. The extract was filtered using a cheese cloth and then using Whatman #1 filter paper. The filtrate was concentrated using a rotary evaporator (Rotavapor® R-210) at 42- 47°C.

2.3. Animals and Treatment

Male Wistar rats aged eight weeks old, weighing 160± 180 g were obtained from the animal facility of the Department of Anatomy at Ladoke Akintola University of Technology, Ogbomoso, Nigeria. The animals were kept in polypropylene cages under room temperature (25°C), in a cycle of twelve hours of light and twelve hours of dark, and were allowed to acclimatize for two weeks. The animals were fed with grower's mash (Farm support services Ltd, Ogbomoso, Nigeria) at a recommended dose of 100 g/kg as advised by the International Centre of Diarrheal Disease Research, Bangladesh (ICDDR, B) daily. They had access to water *ad libitum*. The animals were randomly assigned into four groups of five rats each consisting of (group A control) which received only 2 mL/kg of normal saline and the treatment groups (B, C, D) receiving the doses of 100, 300 and 500 mg/kg body weight of the *A. barteri* extract daily for twenty-eight days, respectively. Twelve hours after the administration of the last *A. barteri* dose, the rats were at the time of sacrifice. They were first weighed, blood samples were collected through ocular artery and centrifuged at 1,500 g/min at 4 °C for ten minutes to obtain serum. The animals were then sacrificed under high ether anaesthesia. All experimental protocols followed the guidelines for Care and Use of Laboratory Animals in Biomedical Research of the National Institutes of Health of the United States (NIH, 1985).

2.4. Measurement of Sperm Parameters

The rats were anaesthetized with diethyl ether. A scrotal incision was made to exteriorize the testis and epididymides. The caudal epididymis was carefully removed, blotted free of blood and was then placed in a pre-warmed Petri dish containing 1.0 mL of physiological saline solution (maintained at 37°C). Several incisions were made on it to allow sperm swim out. Semen analysis was carried out immediately using the new improved Neubauer's haemocytometer counting chamber for determination of the concentration of spermatozoa. Sperm motility was also assessed immediately by counting both motile and immotile spermatozoa per unit area at the magnification of x40. Sperm viability was assessed using eosin-nigrosin test. The percentages of unstained (live) and stained (dead) spermatozoa were calculated by counting 200 spermatozoa per sample. The morphological appearance of normal and abnormal spermatozoa was determined by examining stained smears under the oil immersion (100x) and their percentages were calculated. (WHO, 1999)

2.5. Hormonal Analysis

Hormonal profile of the following endocrine markers (Testosterone TT, Follicle stimulating hormone FSH, and Luteinizing hormone LH) were measured using commercially available immunoassay (ELISA) method (Randox Laboratories Ltd, Admore Diamond Road, Crumlin, Co., Antrim, United Kingdom, Qt94QY) in accordance with the manufacturer's instructions.

2.6. Malondialdehyde Concentration Measurement in Serum

Free radical damage was determined by specifically measuring malondialdehyde (MDA). The MDA, formed as an end-product of lipid peroxidation (LPO), was treated with thiobarbituric acid to generate a colored product measured at 532 nm (MDA detection kit, Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

2.7. Super Oxide Dismutase Activity Measurement in Serum

The activity of SOD was measured by following the method of Beyer and Fridovich (1987).

2.8. Glutathione Peroxidase Activity Measurement in Serum

The GSH peroxidase and GPX activity were quantified by following the decrease in absorbance at 365 nm induced by 0.25 mM H_2O_2 in the presence of reduced GSH (10 mM), nicotinamide adenine dinucleotide phosphate (NADPH) (4 mM), and 1 U enzymatic activity of GSH reductase (GR) (Yoshikawa *et al.*,1993).

2.9. Catalase Activity Measurement in Serum

Serum CAT activity was determined according to the method of Beers and Sizer, as described by Arash, 2015, by measuring the decrease in absorbance at 240 nm due to the decomposition of H_2O_2 in a UV recording spectrophotometer. The reaction mixture (3 mL) contained 0.1 mL of serum in phosphate buffer (50 mM, pH 7.0) and 2.9 mL of 30 mM H_2O_2 in phosphate buffer (pH 7.0). An extinction coefficient for H_2O_2 cm-1 was used for calculation. The specific activity of CAT was expressed as moles of H_2 reduced per minute per mg protein, at 240 nm. An amount of 40.0 M-1 cm-1 was used for calculation. The specific activity of CAT was expressed as moles of H_2O_2 reduced per minute per mg protein.

2.10. Statistical Analysis

Data were expressed as Mean ± SEM. Statistical differences between the groups were evaluated by one-way ANOVA, followed by the Dunnets comparison test to compare the treated groups to the control groups. Differences yielding $p<0.05$ were considered statistically significant. All statistical analysis of data was performed using GraphPad Prism 5.0 for Windows (GraphPad Software, San Diego, California, USA).

3. Results

3.1. Body Weight and Accessory Glands Weight

At the end of the experiment (twenty-eight days), there was an increase in the relative body weight of the *A. barteri* extract-treated groups when compared with the control groups. The absolute weight of Testes, Epididymis, Ventral prostate, Seminal vesicle and Vas deferens of the *A. barteri* extract-treated rats, significantly ($p<0.05$) increased in comparison to the control groups (Table 1).

3.2. Sperm Parameters

The *A. barteri* extract significantly ($p<0.05$) increased the sperm motility and sperm count (68.40±2.48, 73.52±2.53, 77.61±2.49) and (65.78±1.44, 71.21±1.92, 75.16±2.50) in the extract- treated rats when compared with the control (64.60±2.67) and (56.13±13). However, there was no significant difference in the percentages of abnormal sperm cells (morphology) in the treated groups (25.20±1.02, 25.80±1.63, 21.75±1.10) in comparison to the control groups (24.50±1.00) after twenty-eight days of administration (Table 2).

3.3. Serum Testosterone, Follicle Stimulating Hormone and Lutenizing Hormone

The administration of *A. barteri* extract for twenty-eight days significantly ($p<0.05$) increased the serum total testosterone in the treated animals compared to controls. The results were 0.18±0.01, 0.23±0.03; 0.29±0.04 and 0.17±0.01 for the *A. barteri* extract group and controls respectively. The extract produced a non-significant decrease in the blood level, of Follicle Stimulating Hormone (FSH) of the all treated groups (0.14 ± 0.50, 0.12 ± 1.00, 0.11±2.01) when compared to the value of the control group (0.15±0.20). Also, there was a non-significant increase in the level of luteinizing hormone in the treated groups (0.12±0.03, 0.13±0.01, 0.11±0.03) when compared to the control (0.10±0.01) (Figure 1).

3.4. Malondialdehyde (MDA) and Antioxidant Enzymes

The administration of *A. barteri* extract for twenty-eight days significantly decreased MDA concentration in the experimental group compared to the controls ($P<0.05$), with 2.81±0.07, 2.43±0.13, 1.80±0.19 and 5.37±0.41, respectively. Also SOD concentration significantly increased in the experimental group compared to the control group ($P<0.05$), with the results of 1140±48.61, 1336.00±39.10, 1498.00±58.10 and 922.60±36.89, respectively. The extract produced a significant increased GPX concentration in the experimental group (139.00±1.33, 143.70±1.76, 150.80±2.66) compared to the control group (124.30±2.05) (P <0.05). Furthermore, the oral consumption of *A. barteri* extract for twenty-eight days significantly (P <0.05) increased serum catalase activity in the experimental group (332.90±8.18, 346.60±6.57, 363.90±7.12) in comparison to the control group (303.30±9.61) (Table 3).

Table 1. Effect of *Alafia barteri* extract on body and reproductive organ weights of male wistar rats.

Parameters	Groups			
	A (2 mL/kg) control	B(100mg/kg bw)	C(300mg/kg bw)	D(500mg/kg bw)
Initial Body Weight (g)	166.70±70	169.00±1.72	167.20±1.71	167.50±1.11
Final Body Weight (g))	190.60±3.64	193.50±3.40	188.60±3.42	191.50±3.52
Testes Absolute weight (g)	1.88±0.08	2.18±0.08*	2.37±0.09*	2.50±0.03*
Epididymis Absolute weight (g)	0.36±0.02	0.46±0.02*	0.48±0.01*	0.57±0.01*
Ventral prostate Absolute weight (g)	0.28±0.08	0.37±0.04*	0.42±0.01*	0.48±0.01*
Seminal vesicle Absolute weight (g)	0.46±0.01	0.53±0.01*	0.60±0.01*	0.62±0.02*
Vas deferens Absolute weight (g)	0.13±0.01	0.12±0.01	0.14±0.01	0.15±0.02*

Values are expressed as Mean ± S.E.M, n=5 in each group, * represent significant dissimilarly from the control group at $p < 0.05$. One-Way ANOVA. bw: body weight. A: 2 mL/kg normal saline, B: 100 mg/kg bw *A. barteri*, C: 300 mg/kg bw *A. barteri*, D: 500 mg/kg bw *A. barteri*

Table 2. Effect of *Alafia barteri* extract on sperm characteristics of male wistar rats**.**

Groups	Parameters				
				Morphology (%)	
	Sperm motility (%)	Sperm count (x10^6/mL)	Viability (%)	Normal	Abnormal
A (2mL/kg)control	64.60±2.67	56.13±13	57.87±0.71	75.50±1.09	24.50±1.00
B (100mg/kg bw)	68.40±2.48	65.78±1.44*	58.49±0.84	74.80±1.02	25.20±1.02
C (300mg/kg bw)	73.52±2.53	71.21±1.92*	62.01±0.83*	74.20±1.63	25.80±1.63
D (500mg/kg bw)	77.61±2.49*	75.16±2.50*	65.07±0.97*	78.25±1.10	21.75±1.10

Values are expressed as Mean ± S.E.M, n=5 in each group, * represent significant dissimilarly from the control group at $p < 0.05$. One-Way ANOVA. bw: body weight. A: 2 mL/kg normal saline, B: 100 mg/kg bw *Alafia barteri*, C: 300 mg/kg bw *Alafia barteri*, D: 500 mg/kg bw *Alafia barteri*

Table 3. Effect of *Alafia barteri* extract on malondialdehyde (MDA) and antioxidant enzymes.

Parameters	Groups			
	A (2 ml/kg normal saline)control	B (100 mg/kg bwt Ab)	C (300mg/kg bwt Ab)	D (500 mg/kg bwt Ab)
Malondialdehyde	5.37±0.41	2.81±0.07*	2.43±0.13*	1.80±0.19*
Super oxide dismutase, u/g Hb	922.60±36.89	1140±48.61*	1336.00±39.10*	1498.00±58.10
Glutathione peroxidase, u/mg Hb	124.30±2.05	139.00±1.33*	143.70±1.76*	150.80±2.66*
Catalase, u/mg Hb	303.30±9.61	332.90±8.18*	346.60±6.57*	363.90±7.12*

Values are expressed as Mean ± S.E.M, n=5 in each group, * represent significant dissimilarly from the control group at $p < 0.05$. One-Way ANOVA. bwt: body weight, Ab: *Alafia barteri.* A: 2 ml/kg normal saline, B: 100 mg/kg bw *Alafia barteri*, C: 300 mg/kg bw *Alafia barteri*, D: 500 mg/kg bw *Alafia barteri*

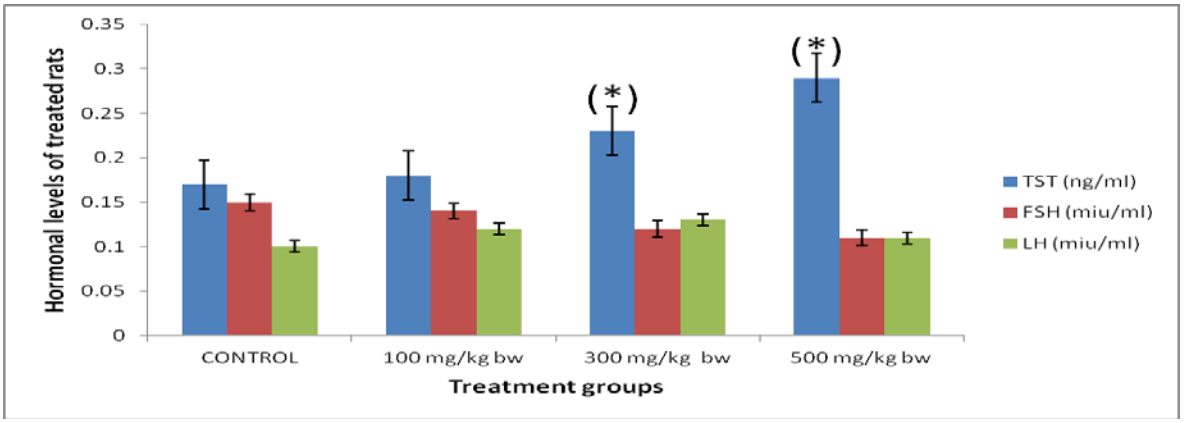

Figure 1: Effect of *Alafia barteri* extract on serum Testosterone, Follicule stimulating hormone and Luteinizing hormone of male wistar rats. Values are expressed as Mean ± S.E.M, n=5 in each group, * represents significant dissimilarly from the control group at $p < 0.05$. One-Way ANOVA. TST: Testosterone, FSH: Follicle stimulating hormone, LH: Luteinizing hormone, Miu: Milli international unit, ng: Nano Gram. bw: body weight. A: 2 mL/kg normal saline, B: 100 mg/kg bw *A. barteri*, C: 300 mg/kg bw *A. barteri*, D: 500 mg/kg bw *A. barteri*

4. Discussion

Medicinal plants are used in the treatment of several diseases attributed to the presence of antioxidants. (Virgili *et al.*, 2001).

The Impact of *Alafia barteri* (*A. barteri*) Oliv., Apocynaceae leaves extract on spermatogenesis and steroidogenesis functions of rats' testis have not been studied. The current study investigated the effects of the leaf extract of *A. barteri* on spermatogenesis and steroidogenesis in order to elucidate its possible implications that could occur following its following consumption. The effect of the extract of *A. barteri* shown in this study revealed a potential increase in mean body weights and weights of reproductive organs (Table 1). Previous observation revealed that androgens regulate the weight, size and secretory function of testes, epididymes and accessory organs (Choudhary and Steinberger, 1975). In addition, This finding is consistent with the findings of Shittu *et al*., (2007) which showed that increased cellular activities are key factors to be considered in the evaluation of organ weights.

The results of the current study reveal the impact of *A. barteri* on spermatogenesis in a dose- dependent manner. Results also shows improved sperm concentration, motility, percentage normal and abnormal morphology sperm of the groups of animal treated with *A. barteri* extract for the duration of twenty-eight days in comparison to the control group rats. This showed that the administration of *A. barteri* extract successfully increased sperm qualities. It has been reported that *A. barteri* is rich in antioxidant constituents such as total polyphenols, flavonoids, tannins, alkaloids, saponnins, vitamin E, vitamin C and vitamin A (Lasisi *et al.*, 2016). Therefore, it is plausible to deduce that the rich antioxidant constituent of *A. barteri* boosted the testicular non-enzymatic and enzymatic antioxidants to effectively scavenge the free

radicals preventing lipid peroxidation as reflected in the increased sperm count and sperm motility. This finding is consistent with the reports of Rodrigues *et al*.,2005 and Bansal and Bilaspuri, 2011. Furthermore, Herbal antioxidants eliminate and suppress ROS formation; the reduction of ROS is a crucial factor in the production of sperm cells and optimization of the fertility rate (Khaki *et al.*, 2010; Henkel, 2005). The administration of *A. barteri* in this study has increased glucose metabolism leading to the production of pyruvate which is known to be the preferred substrate essential for the activity and survival of sperm cells (Dua and Vaidya, 1996; Egbunike *et al*., l986). Although pathophysiology of male infertility has always been unclear, evidence shows that antioxidative changes are probably responsible for the abnormal spermatozoa function and fertilization capacity (Akinloye *et al.*,2005). Seminal plasma, which is a rich source of antioxidants, protects sperm against oxidative stress by several enzymes with a powerful antioxidant activity such as CAT, SOD and GPX. According to previous studies, a decreased level of antioxidants in seminal plasma of infertile men is correlated with the elevated level of MDA, which results in important LPO (Arash, 2015). GPX is an important antioxidant, which protects the epididymis and the ejaculated spermatozoa (Sunde, 1984). This study showed that the administration of *A. barteri* extract as an antioxidant increased SOD, GPX, and CAT, leading to the elimination of ROS thereby protecting sperm cells from oxidation. In addition, *A. barteri* has the potential to restore fertility and normal spermatogenesis, and to improve hormonal levels and sperm quality with concomitantly decreasing the MDA level.

It could be inferred in this present study that antioxidants such as flavonoids and vitamins present in *A. barteri* extract supported sperm morphology, sperm survival, and sperm function which could be regarded as a regular supply of additional nutrients to the treated rats in comparison with the control groups. The improved sperm quality in the treated groups suggests that the *A. barteri* extract can generate simulative influence on hypothalamus. Although the current study shows that there was a decrease in the blood level of follicle-stimulating hormone, the increase in the level of serum testosterone and luteinizing hormone of the treated rat groups indicates the positive impact of *A. barteri* extract in rats. This finding is consistent with a previous study which revealed that antioxidants improve steroidogenesis by enhancing the primary effect of the leydig cell endocrine function in addition to increasing the circulatory testosterone production and the stimulation of spermatogenesis (Prasad and Rajalakshnmi, 1989). Moreover, spermatogenesis and male fertility depend on the presence of testosterone in testis. The reduction of serum testosterone is presumably associated with changes in the body composition, muscle strength, diminished energy, and sexual function. Therefore, androgens and partial androgens are important for the maintenance of spermatogenesis and male fertility (Vahdani and Khaki, 2014), especially in various diseases which exacerbate the decline in testosterone.

5. Conclusion

This investigate on confirmed that the oral administration of the leaf extract of *A. barteri* has no toxic or disruptive interference with spermatogenesis and steroidogenesis in wistar rats. The present study revealed that the oral administration of the *A. barteri* extract possesses profertility properties. These profertility properties can be exploited really well in male fertility therapy.

Conflicts of interest

The authors declare no conflicts of interest.

Acknowledgments

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Jordan Journal of Biological Sciences **Short Communication**

Differential Gene Expression of Durum Wheat (*Triticum turgidum* L. var. durum) in Relation to Genotypic Variation under NaCl Salinity Stress

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Abstract

Variation in expression of important genes under environmental stress is suggested to play an important role in determining phenotypic diversity. Virtually little is known about the genotypic-specific expression variation in durum wheat as an important cereal plant. This study examines transcript levels in durum (*Triticum turgidum* L.) using real-time PCR analysis in relation to genotypic differences by contrasting two gene pools of durum wheat under NaCl salinity stress. Four important genes in relation to stress were analyzed, three of which showed differential down-regulation of expression at the RNA level. Specifically, it is found that the expression of *GAD* and *ASN* genes showed a different pattern in response to different levels of salinity. At 50 mM NaCl *GAD* was down-regulated in only (Sham 1) genotype, while *ASN* was down-regulated in (Sham1), (Um Qais), and (Akcad). Under 100 mM NaCl, both genes were down-regulated in the genotype (Um Qais), while salinity of 150 mM NaCl induced down-regulation of both genes in (Um Qais) and (Norseh). Genetic variation (cultivars vs. landraces) was found to show a significant relationship to salinity responses especially at 50 and 150 mM NaCl (one-way ANOVA; $P < 0.05$). *SOD* and *CAT* genes were also down-regulated by salinity, but the relationship to genetic variation seems to be statistically less important. The genetic difference in gene expression could be best attributed to transcriptional and post-transcriptional regulation processes that are involved in durum tolerance to stress. This work demonstrates that the durum is an excellent plant system to study genetic variation in relation to gene expression under environmental stress because it seems that alleles of different genotypes of durum are affected by genetic background or environmental factors. The results of this study may help to classify the studied genotypes into sensitive and tolerant varieties.

Keywords: Durum, qRT-PCR, Gene expression, Genetic difference, NaCl, Wheat, Jordan.

1. Introduction

Durum or pasta wheat (*Triticum turgidum* L. var. durum, 2n = 4x = 28; AABB genomes) is an important plant grown for a long time in the Middle and Near Eastern countries (Jaradat and Duwayri 1981; Belaid 2000). Durum production is largely determined by the habitat conditions such as drought and soil salinity (Munns *et al*., 2006; Carvalho *et al*., 2011). Genetic variation, relatively abundant, in durum wheat seems to be a main reason for the adaptation of this cereal in salt-affected areas especially in dry and semi-dry soils. Durum contains a high level of genetic variation that includes nucleotide polymorphisms and large variants of DNA fragments and polymorphisms (Elía *et al*., 2016; Laddomada *et al*., 2017) . Genetic variation is believed to influence the pattern and level of gene expression especially under environmental heterogeneity of stress (Saintenac *et al*., 2018). It has been shown that different genotypes of the same species are not expressed equally at the transcript level, and such variation is linked to important regulatory mechanisms involved in stress tolerance and adaptation to stress factors, such as soil salinity (Müller *et al*., 2018).

Soil salinity is an important factor that reduces plant growth and productivity in several parts of the world, particularly arid and semiarid regions (Ren *et al*. 2005). Salinity can potentially induce changes in genetic variation in transcript pattern and levels of various regulatory mechanisms including transcriptional and post-transcriptional regulations (Tirosh *et al*., 2006).

Salinity has been shown to cause osmotic and water stress by increasing the accumulation and the up-regulation of important enzymes that largely show differential responses in varied genotypes of a species (Zhand *et al*., 2017). This indirect influence of genetic variation through the modulation of gene expression can ultimately lead to phenotypic variation (AL-Quraan *et al*., 2010; Giunta *et al*., 2018). The response to salinity typically involved both enzymatic and no-enzymatic antioxidants defense mechanisms (Sharma *et al*., 2012). These mechanisms play a role in combating the accumulation of reactive oxygen species (ROS) by a

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diverse set of enzymes such the superoxide dismutase (SOD), which dismutase the O_2^- radicals to H_2O_2 (Luis *et al*., 2018). Additionally, catalase (CAT), glutamic acid decarboxylase (GAD), and asparagine synthetase (ASN) are among the other enzymes that scavenge H_2O_2 and reduced its accumulation to toxic levels. These enzymes were shown to have different reactivity to H_2O_2; CAT, for example, has low affinity to H_2O_2, and it was shown that it is important for scavenging most of the H_2O_2 (Harb and Samarah 2015; Duan *et al*., 2018; Klein *et al*., 2018;).

Alterations in gene expression is an important defensive response to salinity (Rabbani *et al.*, 2003). Variation in gene expression in various plant species such as in maize (Wang *et al*., 1999) and the tomato (*Lycopersicon esculentum*) (Cong *et al*., 2002) has been shown to be related to plant species. However, studies on the response of different genotypes of the same species to salinity response in relation to genetic variation and gene expression are limited. Accordingly, this study used quantitatively real-time PCR to evaluate genetic variation in relationship to gene expression of six local durum genotypes of two genepools, i.e., landraces and cultivars.

2. Materials and Methods

2.1. Durum Seeds and NaCl Treatments

Durum wheat seeds of six Jordanian varieties (Um Qais, Norseh, Dar Alla, Sham 1, Hourani, Akcad) were obtained from the National Center for Agricultural Research and Extension (NCARE) of Jordan. The studied varieties of wheat are of two genepools: cultivars (Deir alla 6, Acsad 65, and Sham 1) and local landraces (Um-qais, Hourani 27, and Noorseh). Seeds of a uniform size of each genotype were utilized in this study. The seeds were surface-sterilized with 70 % (v/v) ethanol and then with 2 % commercial bleach (sodium hypochlorite) for 3-5 minutes, and finally rinsed thoroughly in sterile distilled water. The seeds were then subjected to a stratification for a few days at approximately 4°C. Seeds from each genotype were germinated on 12 × 12 cm petri dishes using a wet filter paper (Whatman No 1, Kent, UK) at 25°C in dark conditions with or without salt treatment at four NaCl levels (0 mM as a control, 50 mM, 100 mM, 150 mM). The four levels of salinity were chosen as they have been shown to cause several growths and physiological responses to cereal cultivars (Salama *et al*. 1994). After three days of treatment, leaves from the wheat genotypes were collected, and were quickly frozen in liquid nitrogen at -20°C until RNA isolation.

2.2. RNA Isolation and cDNA Synthesis

RNA was isolated from wheat tissues using an IQeasy™ Plus plant RNA isolation kit (iNtRON Biotechnology, Korea). The concentration of isolated RNA in the samples were estimated spectrophotometrically (260 nm/280 nm; Biochrom, Cambridge, UK). The first-strand cDNA was synthesized by mixing 2 µg of RNA with 4 µL of Prime Script™ RT reagent (Takara, Japan), and the final volume of the mixture was adjusted to 20 µL with RNase-free water (0.1% (v/v); diethylpyrocarbonate-treated water). The mixture was then placed in a thermocycler (Biometra, Germany) for forty-five minutes at 37°C, followed by fifteen seconds at 85°C, and finally at 4°C for approximately five minutes. The resultant amplified products were then diluted to 50 ng/µL with sterile RNase-free water and stored at -20°C for gene expression analysis by quantitative real-time PCR (qRT-PCR).

2.3. Primer Design and Quantitative Real-Time PCR

qRT-PCR was used to examine the relative expression of the four genes in durum wheat genotypes after exposure to different levels of salinity. The gene-specific primers for qRT-PCR were designed on the basis of the sequences existent in GenBank using the software Primer3 (http://bioinfo.ut.ee/primer3-0.4.0/primer3/input.htm) as described in Thornton and Basu (2011). The primers used showed a high specificity as revealed by gel electrophoresis. Differential gene expression analysis of (CAT, GAD, ASN, SOD) genes was studied using qRT-PCR. The PCR reactions were made in a volume of 25 µL prepared from 10 µL of Kappa Syber Fast qPCR reagent (KAPA Biosystems, USA), 50 ng/µL template cDNAs, and 10 µM aliquots of each primer of the genes (Table 1). The cycling conditions utilized were: 2 min/95°C, 10 s/ 95°C, 25 s/57°C, 25 s/60°C, and a final extension step of 2 min/60°C using a CFX96 touch real-time PCR system (Bio-Rad) machine. The experimental procedure was conducted at least three times. The threshold cycle (Ct) values of the triplicate PCRs were averaged, and the relative quantification of the transcript levels was analyzed using the comparative Ct method (Schmittgen and Livak 2008).

2.4. Statistical Analysis

Statistical analysis was done with the help of SPSS for Mac (v20, SPSS Inc., Chicago, IL, USA). The differential response to salinity for the studied genes were estimated by one- and two-way analyses of variance (ANOVAs) and Duncan's Multiple-Range test (DMRT). The values shown in the results are the arithmetic mean ± standard error (S.E.; n=3), with p value ≤ 0.05 regarded as a statically significant difference. Before the analysis, normality was checked (Shapiro-Wilk test), and when normality and homogeneity of variance were violated, the logarithmic transform was taken for the raw data. To measure the level of gene expression of all genes, the actin gene was used as an internal control to quantify the relative transcript level of each target gene (Goidin *et al*., 2001).

Table 1. Primer sequences, accession numbers and the amplicon size of the primers used in this study.

Gene name	Sequence from 5' to 3 '	Gene Bank accession #	Amplicon size (bp)
ASN-F/R	TGTCCTTTATACATGGGCTGGG/ ATCATAAGGGGCTGAGGGAATG	KC193248.1	192
Cat-F/R	AACTTCCCCGTCTTCTTCATCC/ AGAGGAAGGTGAACATGTGGAG	KP696753.1	154
GAD-F/R	ATCTTCCACATCAACTACCTCGG/ TTCTCCATGATGTTCCTGTACCC	AK355055.1	137
MnSOD-F/R	TGCTGCTTTACAAGGATCTGGA/ TCCCAGACATCAATTCCCAACA	KP696754.1	144
Actin-F/R	CTCCATCATGAAGTGTGACGTG/ GACGACCTTGATCTTCATGCTG	AY145451.1	151

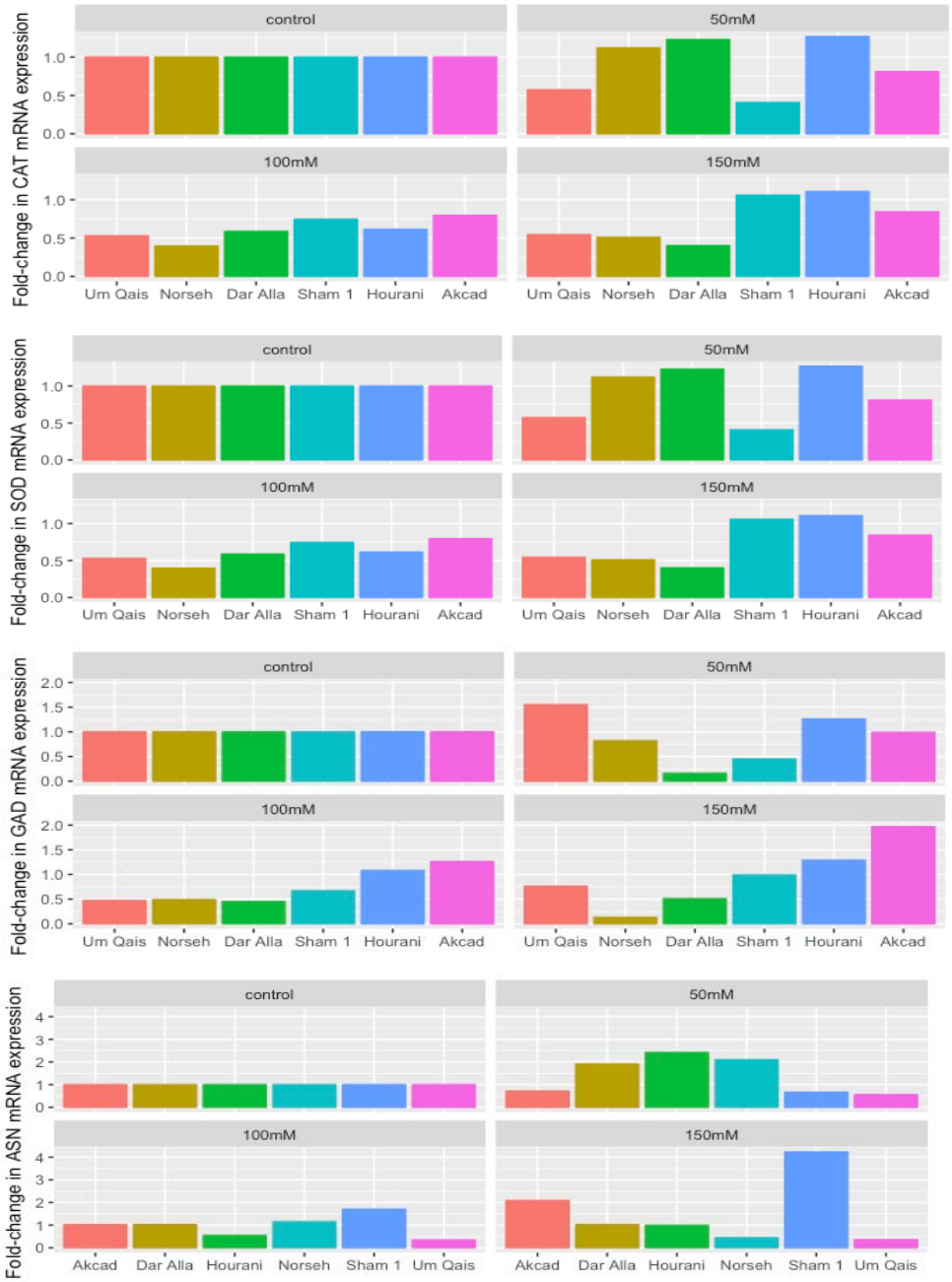

Figure 1. Relative expression levels of the four genes studied in six durum genotypes exposed to different concentrations of NaCl salinity. Fold changes greater than one (> 1) indicate up-regulation, and (< 1) down-regulation.

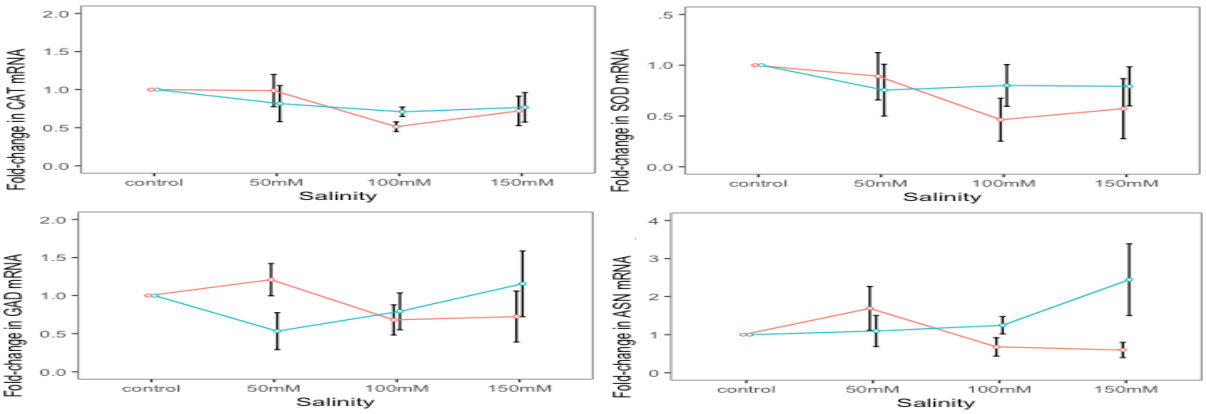

Figure 2. The relative expression levels of the four genes studied in landraces (Um-qais, Hourani 27, and Noorseh; blue line) genotypes and cultivars (Deir alla 6, Acsad 65, and Sham 1; orange line) exposed to varied salinity levels.

3. Results and Discussion

The present study investigates the expression levels of four important genes involved in a wide range of physiological and biochemical processes that are important for the tolerance of plants against environmental stresses (Herzog *et al*., 2018). Results present in this study revealed that at 50 mM NaCl the *CAT* gene was down-regulated in (Um Qais), (Sham 1), and (Akcad). Under 100 mM NaCl (Um Qais), (Norseh), (Dar alla), (Sham 1), and (Akcad) genotypes were found to be down-regulated, whereas at 150 mM NaCl induced gene down-regulation in the genotypes (Um Qais), (Norseh), (Dar Alla), and (Akcad) (Figure 1). This variation in gene expression of *CAT* in different genotypes of durum wheat may suggest that the genotypes (Um Qais), (Dar Alla), (Akcad) seem to be more sensitive to salt compared to the other studied genotypes. The expression levels of *SOD* gene were largely similar to *CAT* gene in all studied genotypes. Specifically, under 50 mM NaCl the *SOD* was down-regulated in (Um Qais), (Sham 1), and (Akcad), and under 100 mM NaCl the genotypes SOD was found to be down-regulated in (Um Qais), (Norseh), (Dar alla), (Sham 1), and (Akcad). However, the high concentration of salinity, i.e., (150 mM NaCl), induced downregulation of *SOD* in the genotypes (Um Qais), (Norseh), (Dar Alla), and (Akcad) (Figure 1). The similar expression pattern of *CAT* and *SOD* genes indicated that salinity of different levels induce similar responses in durum wheat independent of their genetic background. In fact, one-way ANOVA did not signify any important difference ($P > 0.05$; Figure 2) between cultivar or landrace nature of the genotypes. Moreover, these results perhaps suggest that both *CAT* and *SOD* genes are involved in rather similar salinity tolerance mechanisms. These results are similar to those found in barley and wheat in some defense-related genes (Herzog *et al*., 2018; Karlik and Gözükırmızı 2018), implying that the expression of the *CAT* and *SOD* genes are closely related to salt tolerance, and thus may play an essential role in plant defense.

The expression of *GAD* and *ASN* genes showed a different pattern in response to varied concentration of salinity. Under 50 mM NaCl *GAD* was down-regulated in only (Sham 1) genotype, while *ASN* was down-regulated in (Sham1), (Um Qais), and (Akcad) (Figure 1). Under 100 mM NaCl both genes were down-regulated in the genotype (Um Qais), while salinity of 150 mM NaCl induced down-regulation of both genes in (Um Qais) and (Norseh). Perhaps, this suggests that (Um Qais), (Sham 1), (Norseh), and (Akcad) seem to be more sensitive compared to (Dar alla) and (Horani) at all salinity concentrations possibly due to their genetic variation. In fact, genetic variation (cultivars vs. landraces) was found to show a significant relationship to salinity responses especially at 50 and 150 mM NaCl (Figure 2).

Additionally, the results of this study showed an up-regulation pattern of gene expression in some of the studied genes in relation to genetic variation and in response to salinity. Namely, the genotypes (Dar alla), (Norseh), and (Horani) showed up-regulation expression of the genes *CAT*, *SOD*, *ASN* under salinity of 50 mM NaCl, and under 150 mM NaCl (Sham 1) and (Horani) showed up-regulation of the same genes, i.e., *CAT*, *SOD*, and *ASN* (Figure 1). On the other hand, the 100 mM NaCl induced up-regulation of *ASN* and *GAD* only in (Sham 1), (Akcad), and (Horani). This genetic difference in gene expression could be resulting from a number of regulatory processes, including, for example, transcriptional and post-transcriptional regulations. Moreover, variation of the expression of the studied genes in this study could be also attributed to the decreased level of transcript abundance caused by salinity (Ramirez-Parra *et al*., 2004; Zhand *et al*., 2017).

In summary, this study shows that the gene expression variation occurred differentially in durum wheat in response to different levels of salinity genetic background. Although only few genes were analyzed, distinct expression patterns were found between a landraces and cultivars. This work demonstrates that the durum is an excellent plant system to study genetic variation in gene expression under environmental stress. It can be concluded then that gene expression in durum wheat is differentially determined by the combined effects of genetic and environmental factors.

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