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EDITORIAL PREFACE

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

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

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

December, 2015

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

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Cryopreservation and Genetic Stability Assessment of Threatened Medicinal Plant (*Ziziphora tenuior* L.) Grown Wild in Jordan

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Abstract

Ziziphora tenuior L. is one of the important medicinal plants that belong to the *Lamiaceae* family. It is a rare species with a promising medicinal potential and grows wild in the southern part of Jordan. Unfortunately, this plant might be totally extinct from the wild due to over-exploitation. Two cryopreservation techniques (encapsulation-dehydration and encapsulation- vitrification*)* were applied for *in vitro* conservation of this valuable medicinal plant, and after that the explants were tested for their genetic stability using Amplified Fragment Length Polymorphism (AFLP) technique. In the encapsulation-dehydration experiment, the results revealed that 40% of the cryopreserved shoot tips survived when they were dehydrated chemically on 0.75 M sucrose in MS supplemented media for one day and exposed to air dehydration for 6 hrs. Moreover, the best recovery rate (20%) was obtained when either 0.5 M or 0.75 M sucrose MS supplemented media were used as preculture media for the shoot tips for one day, followed by air dehydration for 4 or 6 hrs. Meanwhile, in the encapsulation -vitrification experiment, the highest survival (37.5%) and recovery (10%) percentages of the cryopreserved shoot tips were obtained when the encapsulated shoot tips were pretreated for 60 min. with the loading solution before being exposed to PVS2 vitrification solution and LN. AFLP technique had clearly showed that, there were no genetic variations between the shoot tips of *Ziziphora tenuior* L., before and after cryopreservation.

Keywords: Cryopreservation, Encapsulation-dehydration, Encapsulation- vitrification, *Ziziphora tenuior* L.

1. Introduction

Ziziphora tenuior L. is one of the important species that belong to the family Lamiaceae. It is widely spread in many countries around the world, such as China, Iran, Afghanistan and Iraq (Zargari, 1995). *Ziziphora tenuior* L. is also distributed in Jordan in Steppes, Al-Nagab desert, lower Jordan valley, Araba valley, Amman, Madaba and Edom (Zohary and Feinbrun, 1978). Also, according to Al-Rawashdeh, (2011), *Ziziphora tenuior* L. is spread particularly in the southern part of Jordan mainly at Alshoubak region (Al-Rawashdeh, 2011). This plant is distinguished for its valuable medicinal potential as it has been used in folk medicine in many treatments (Ozturk et al., 1995). For example, it was used for the treatment of dysentery, fever, diarrhea, coughing, bladder stone, painful menstruation, stomach tonic and abortifacient (Naghibi et al., 2005). Furthermore, it is used in making herbal tea due to its odors (Al-Rawashdeh , 2011). In Jordan, few studies had researched Ziziphora tenuior. For example, *Ziziphora tenuior* was undergone molecular taxonomy using RAPD technique, and the result of this study confirmed that there is a variation between this plant and Mentha sp. (Al-Rawashdeh, 2011). The other study that highlighted Ziziphora tenuior was conducted by Oran (2013) who searched for the flowering plants that grow wild in Tafila Province, and reported that this genetic resource is under threats of extinction. This was due to several reasons including illegal collection, agricultural expansion and urbanization, deforestation, overgrazing, soil erosion, and depletion of water resources (Oran, 2013). So, there is a great need for protecting and conserving this important genetic resource to insure its availability for scientific research and phytoindustry.

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Biotechnology is an integral part of international plant conservation programs and plays a role in preserving plant genetic resources. Cryopreservation is a part of biotechnology (Bajaj, 1995; Benson, 1999) and during the last 25 years, cryopreservation was proved to be the most precious method for biological materials long-term conservation. Cryopreservation is simple and applicable to a wide range of genotypes (Engelmann, 2004). Cryopreservation is considered safe, cost-effective and strategy for long-term preservation of biological materials (Reed, 2008; Shibli et al., 1999b). Among the advantages of cryopreservation are that it has low maintenance cost, allows small storage space requirement and there is no need for many replicates to conserve a plant (Shibli and Al-Juboory, 2000). Moreover, cryopreservation has been used for storing many dissimilar types of plant material, such as embryonic axes isolated from seeds, seeds, and vegetative propagated plant material, including pollen, apical or auxiliary buds, embryogenic tissues and somatic embryos (Salaj et al., 2011; Teresa et al., 2010). Hypothetically, using cryopreservation storage plant materials could be preserved without changes for unlimited periods (Wen and Wang, 2010).

Regeneration and genetic stability potential of cryopreserved plant materials are maintained for an indefinite period (Al-Ababneh et al., 2003). Problems such as genetic instability and risk of loss biological materials due to contamination or human error during subculture can be overcome (Kaviani, 2011). Due to the possibility of presence of epigenetic alterations after cryopreservation, there was a need to validate plant genetic uniformity of the cryopreserved plant material after exposure to LN (Harding, 2004; Micula et al., 2011). To detect any possible genetic variation in plants material, several molecular techniques have been applied (Harding, 2004). DNA (RAPD) and amplified fragment length polymorphisms (AFLP) were the most popular markers used in this domain (Micula, et al., 2011).

Amplified fragment length polymorphisms (AFLP) technique is widely used as a DNA fingerprinting system for detecting genetic variation among living organisms (Micula, et al., 2011). This technique is distinguished for its high reproducibility and high levels of polymorphism (Hao et al. 2001; Nighat et al., 2010). In addition to the ability of AFLP to permit the simultaneous analysis of many loci spread over the entire genome without need for a prior knowledge of the organisms under study, AFLP proved to be a very sensitive, reliable fingerprinting technique that can be applied to resolve differences between isolates of the same species in a broad range of taxa in most living organisms (Mu¨ ller et al., 2007).

There is no reported literature on the in vitro conservation or genetic assessment of *Ziziphora tenuior* L. which grows wild in Jordan. Therefore, this research was conducted to study the possibility of long-term conservation (cryopreservation) of *Ziziphora tenuior* L. using encapsulation-dehydration and encapsulation-vitrification methods and to test genetic stability of cryopreserved plant material using AFLP technique.

2. Materials and Methods

2.1. Establishing Mother Stock

Ziziphora tenuior L. seeds were originally collected from Al-Hisha, Al-Shoubak region (30° 31′ 53″ N, 35° 33′ 39″ E) southern Jordan. Surface-sterilization of seeds was done by dipping them in 70% ethanol solution for 30s, before soaking in a 20% aqueous solution of 5.4% sodium hypochlorite for 20 min with gentle shaking, followed by three washes with sterilized distilled water under laminar air-flow cabinet. After that, the sterilized seeds were inoculated into test tubes containing Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) without growth regulators. Cultures were left in the growth room conditions in dark at 25±1°C until full germination of seeds. Germinated seedlings were transferred to normal conditions of growth room and maintained under daily light regime of 16-h (photosynthetic photon flux density (PPFD) = 40-45 µ mol. m-2 sec-1) light, 8-h dark at 24 ± 1°C. Cultures were sub-cultured periodically every (4-5weeks) to MS hormone free (HF) medium for mother stock establishment.

2.2. Encapsulation-Dehydration

For shoot tip encapsulation, two different liquid media were prepared from stock solutions, the first medium contained 3% sodium alginate and calcium free-MS. The second medium was prepared by using Murashige and Skoog media that includes (100 mM) of calcium (CaCl2) with of final concentration. Next, *Ziziphora tenuior* L. shoot-tips were dissected in vitro and precultured 3 days on solid MS-HF (hormone free) medium provided with 0.3 M sucrose in dark at growth room conditions. Shoot tips were taken individually by using 1ml sterile micropipette with some alginate solution, and then were soaked into liquid MS-HF medium provided with 100 mM CaCl2 and 0.3 M sucrose to produce beads that had a diameter of about 5 mm. Some produced beads had to be without shoot tips for moisture content determination and all the beads had to be polymerized for 30 min with stirring.

Produced beads were then transferred to MS-HF liquid medium containing 0.5 and 0.75M sucrose and lightly rotated on a shaker for 1 to 3 days, after that the media containing 0.5M or 0.75M sucrose was removed and beads dehydrated under laminar air-flow cabinet on a sterilized filter paper for 0, 2, 4, and 6 hrs, then half of the beads were transferred to 2 ml sterile cryovials and dipped into liquid nitrogen for at least 1 hr at (-196ºC). Next, the cryovials were thawed in a water bath at 38-40ºC for 2-3 min, while the other half of beads was not exposed to liquid nitrogen. Both halves were transferred to a recovery MS media provided with 0.1 M sucrose and preserved for 7 days in dark for growth, and next transferred to normal growth conditions for 7 days. After that, shoot tips were studied for 4 weeks for survival and recovery signs. Survival percentage of cryopreserved or non-cryopreserved shoot tips was determined by using TTC (2, 3, 5-triphenyl tetrazolium chloride) for each treatment, where 50 *Ziziphora tenuior* L. shoot tips from each treatment were placed in 5 cryovials (10 shoot

tips/cryovial) and 1 ml of Tetrazolium (TTC) salt solution 0.5% (w/v) was added to each cryovial and kept in a complete darkness for 16 hours. Survival percentage= (number of red shoots /total number of shoots) × 100%

The presence of color with pink or red in the survived shoot tip is a result of reducing Tetrazolium (TTC) salt solution to formazen using hydrogen ions released from viable plant cells as a result of respiration.

For the determination of beads moisture content, after each dehydration period, a fresh weight of beads was measured; the beads were dried at 80 ºC in an oven for 16 hrs and then reweighed. Moisture Content (MC) was determined by the formula:

MC % = [(Beads fresh weight - Beads dry weight) / Beads fresh weight] x100.

2.3. Encapsulation-Vitrification

Encapsulation of *Ziziphora tenuior* L. shoot-tips in beads was done as mentioned in the encapsulation method previously. The beads were placed in sterilized cryovials for different periods (10, 20, 40, 60 and 80 min) with the loading solution (0.4 M sucrose and 2 M glycerol in HF-liquid MS medium). Next, the loading solution was replaced with full strength plant vitrification solution 2 (PVS2) consisted of 15% ethylene glycol, 30% glycerol and 15% DMSO (w/v) in a hormone free liquid medium provided with 0.4 M sucrose for 20 min at room temperature.

Half of the cryovials was not exposed to (LN) and the other half of the cryovials was soaked in (LN) and stored for at least 1 hr. then they were thawed at 38-40 ºC for 2-3 min. after that the PVS2 solution in both halves (with and without LN) was replaced by unloading solution and washed three times for 30 min. Other testing procedures were done as in the experiment described above.

2.4. Genetic Stability Assessment of Ziziphora tenuior L. using Amplified Fragment Length Polymorphism AFLP technique

Five seeds of *Ziziphora tenuior* L. were collected randomly before the seeds were cultured separately on MS-FH media for full germination in the growth room chamber conditions (as mentioned in mother stock establishment section). After that aseptically germinated seedlings were propagated separately for each seed. Next each seed had its mother stock shoots alone. The encapsulation-dehydration technique was applied for cryopreservation of each seed mother stock shoot tips individually. After that, a sample of each seed mother stock was taken before and after they were cryopreserved to test their genetic stability using AFLP technique as described by (Vos et al., 1995).

2.4.1. DNA Extraction

Two grams of *Ziziphora tenuior* L. plant samples were grinded in liquid nitrogen in 1.5 ml tube. Then about 40 mg of tissues powder were placed in 1.5 ml and 600 µl of Nuclei Lysis solution was added. The solution was then vortexed and then incubated at 65 °C for 15 min. RNase solution 3 µL was added to the cell lysate, and the samples were mixed by inverting the tube two to five times and incubated for 15 min at 37 °C.

Samples were left at room temperature for cooling. Then protein precipitation solution (200µl) was added and vortexed vigorously at high speed for twenty seconds. After that the solution was centrifuged for three min at 13,000-16,000 rpm until the precipitated proteins formed a tight pellet. The layer, which was containing the DNA, was removed and transferred to 1.5 ml tube containing 600µl isopropanol. Next, the solution was mixed by inversion until thread-like strands of DNA form a visible mass and it was centrifuged at 13,000-16,000 rpm for one min. before the supernatant was transferred to another tube and DNA was washed by adding 600 µl 70% ethanol. The tube containing the mixture was inverted for several times then centrifuged at 13,000-16,000 rpm for one min. Then, the ethanol was aspirated by using either a drawn pasteur pipette or a sequencing pipette tip and the tube was overturned on top of clean absorbent paper and the pellet was air-dried for 15 min. Then, about 100µl of DNA rehydration solution and rehydrate were added to tube and incubated at 65°C for one hour. The solution was mixed periodically by quietly tapping the tube. The extracted DNA was stored in a refrigerator at 2-8°C.

2.4.2. Quantification of DNA

Agarose gel electrophoresis (0.7 %) was used to determine the quality and quantity of the DNA extracted from the *Ziziphora tenuior* L. tissues. The size and the intensity of each sample were detected with DNA mass ladder (Promega) 1 kp to determine genomic DNA concentration.

2.4.3. Amplified Fragment Length Polymorphism (AFLP) Analysis

AFLP profiles were obtained according to (Vos et al., 1995). Six selective primers were used for DNA amplification with two labeled EcoRI (AAC IRDye 700 and ACT IRDye800) and five MseI unlabeled primers (CAC, M-pr-CTT, CGT, CAT and CTC). DNA samples were digested with EcoRI and MseI restriction enzymes and proper oligonucleotide adapters were ligated with DNA ligase through incubation at 37ºC for 150 min. Double-stranded adapters were prepared by mixing individual synthetic oligonucleotides: EcoRI-adapter was prepared by mixing 7.0 µL of the top strand oligonucleotide (2µg/µL) with 7.5 µL of the bottom strand oligonucleotide (2µg/µL) in 486.1 µL of TE buffer. MseI adapter was also prepared by mixing 63.5 µL of the top strand oligonucleotide (2µg/µL) with 54.5 µL of the bottom strand oligonucleotide (2µg/µL) in 382 µL of TE buffer.

A pre-amplification step was performed using primers (E-pr-A and M-pr-C) designed to amplify the DNA fragment between the adapter sequence and one additional nucleotide. For quality assessment 10 µL of the reaction was used to run on 1.5% agarose gel and the rest (10 µL) was diluted with 40 µL of TE (10 mM Tris and 1mM EDTA, (pH 8), which was enough for about 30 AFLP-reactions. The diluted reaction was stored at -20ºC. Products of pre-amplified DNA were selectively amplified by using combinations of six primers EcoRI+MseI (E-ACC 700 with M-CTC, M-CTA, and M-CAG) and (E-AAG 800 with, M-CTC, M-CTA, and M-CAG).

A touchdown program was used for performed of PCR: thirty cycles of subsequently lowering the annealing temperature (65 ºC) by 0.7 ºC per cycle while keeping denaturation at 94ºC for 30 seconds and extension at 72 °C for 60 seconds. This was followed by 23 cycles of denaturation at 94ºC for 30 seconds, annealing at 56 ºC for 30 seconds and extension at 72 ºC for 60 seconds. After PCR, four µL of blue stop solution were added directly before storage at -20 ºC.

2.4.4. Polyacrylamide Gels

Products of AFLP reaction were analyzed by using automated sequencer (LI-COR, Inc. Lincolen, Nebraska, USA). For gel electrophoresis of AFLP, 6% polyacrylamide gel was used, 200 ml polyacrylamide were prepared with 84g urea, 30 ml acrylamide / bisacrylamide 40% solution (SIGMA), 20 ml of 10X Trisborate-EDTA (TBE) buffer. Gels were cast at least 60 minutes before use and were focused by pre-run for 25 minutes before loading. As recommended by LI-COR, the pre-running and running electrophoretic conditions were performed at 1500V, 40W, 40 mA, 45 ºC, and 4 scan speed. TBE buffer (1X, 89 mM Tris, 89 mM borate, and 2.2 mM EDTA pH 8.3) was used as the running buffer. The purpose of pre-running was to "warm up" the gel to about 45 ºC, this temperature was maintained throughout electrophoresis, resulting in even heat distribution and good quality fingerprints. Samples of DNA were denatured by heating the resulting mixtures for 5 min at 95 ºC and cooled rapidly on ice before loading the gel. To remove urea precipitate or pieces of gel, gel wells were completely rinsed prior to loading by flushing them with buffer using a 20 cc syringe, then one µL of each denatured sample was loaded in the designated lanes, IRDye 700 and IRDye 800 sizing standard were used to determine band size.

2.5. Experimental Design

In cryopreservation experimental part, all treatments in each experiment were arranged in a Complete Randomized Design (CRD) and 40 shoot tips were used in each treatment. The collected data from the experiments described above were statistically analyzed using SAS 9.2 (Statistical Analysis System, Cary, NC) and the standard error of means were calculated for each treatment in each cryopreservation experiment.

3. Results and Discussion

3.1. Encapsulation-Dehydration

A complete survival of the encapsulated non-cryopreserved *Ziziphora tenuior* L. shoot tips was recorded when shoot tips pretreated with 0.5 M sucrose after dehydration periods of (0, 2 hrs) under laminar air, moisture content of beads MC % ranged from (85%) and (68 %), respectively, for each period of dehydration (Table 1). The recovery percentages in the non-cryopreserved shoot tips showed the greatest values (100, 95%) after (0, 2 hrs) dehydration periods, while the minimum value was recorded after (6 hour) dehydration when the MC% reached to (21%) (Table 1). Similar findings were obtained by Younes (2012) who obtained the full survival percentage (100%) of encapsulated none-cryopreserved *Achillea fragrantissima* shoot tips in pretreated media provided by 0.5 M sucrose with or without (3 hrs) dehydration when incubated for three days.

Meanwhile, the results of the present study revealed that, after cryopreservation, the highest survival percentage (35%) in shoot tips pretreated with 0.5 M sucrose supplemented media for one day was obtained after (6 hrs) dehydration period before LN exposure, while the maximum recovery percentage (20%) was obtained after (4 hrs) dehydration period with MC% of (25.5%). Also, it was found that, beads with MC % of (85%) and (68%) did not recover after (0, 2 hrs) dehydration (Table 1). Obtaining the best recovery at MC level of (25%) is expected due to the fact that, most of plant species explants recovered after cryogenic treatment when their MC was between 17-37% (Englemann, 2004).

Also, the results of the present study recorded (40 %) survival rate in the cryopreserved *Ziziphora tenuior* L. shoot tips recorded when pretreated with on 0.75 M sucrose provided media for one day and air dehydrated for 6 hrs. Also, the best recovery rate (20%) in the cryopreserved explants was obtained on shoot tips pretreated with either 0.5 or 0.75 M sucrose supplemented media for one day fallowed by 4 or 6 hrs air dehydration as shown in Fig. (1) and Table (2). However, our results agree with Younes (2012) who reported that the encapsulated shoot tips of *Achillea fragrantissima* obtained the greatest survival and recovery percentages (40, 20%) when pretreated in media provided by 0.5 M sucrose with (6 hrs) dehydration, but it disagrees with her regarding the best recovery which was obtained in her study after incubation for three days, which is not the case in our study. Meanwhile, Moges, et al. (2004) reported that a complete survival of encapsulated cryopreserved violet (*Saintpaulia ionantha*) shoot tips was obtained when pretreated with 0.3 M sucrose and incubated for two days with (2 hrs) of dehydration. Wang et al. (2002) reported that the greatest recovery of encapsulated shoot tips of *Troyer citrange* was obtained when shoot tips precultured in media provided by different concentrations of sucrose ranged from (0.15 M to 0.29 M). In addition, poor growth was obtained when sucrose concentrations in pretreated media lower than (0.15 M or higher than 0.29 M). Maria et al. (2012) reported that promoting recovery of encapsulation cryopreserved white mulberry (*Morus alba*) shoot tips with pretreated media contains 0.75 M sucrose was more effective than 0.5 M sucrose for either one or three days . Also, Shibli et al. (2009) reported that pretreated *Crocus hyemalis* and *Crocus moabiticus* calli gave the greatest survival and recovery percentages, respectively, when encapsulated with 0.5 M sucrose for two days after (2 hrs) of dehydration and cryopreserved. However, the decline in the survival and recovery rates of *Ziziphora tenuior* might be attributed to the formation of extra-cellular and intra cellular- ice crystals as a result of high moisture content of the cryopreserved tissues (Baghdadi et al., 2010; Plessis et al., 1993).

The moisture content of the encapsulated *Ziziphora tenuior* beads declined with increasing exposure duration to air drying (0, 2, 4, 6 hrs), as shown in (Tables 1-4), and

shoot tips with high moisture content were not able to recover after cryogenic exposure (Tables 1-4). High moisture content was found to cause death of the encapsulated African violet (*Saintpaulia ionantha*) shoot tips that pretreated with 0.1 M sucrose for 2 days before (8 hrs) of dehydration (Moges et al., 2004). Also, Tahtamouni and Shibli (1999) showed that moisture content of wild pear beads pretreated with 0.75 M sucrose declined more than those pretreated with 0.3 M sucrose when exposed to the same dehydration period. Survival and recovery were higher of encapsulated cryopreserved bitter almond *(Amygdalus communis*) shoot tips when dehydrated for 6 hrs than 4 hrs (Al-Ababneh et al., 2003). Moreover, (Halmagyi and Deliu, 2011) found that in redwood (*Sequoia sempervirens* Endl.) shoot tips moisture content was reduced to (27%) when pretreated for (24 hrs) in media supplemented with 0.5 M sucrose followed by (3 hrs) air dehydration which resulted in (67%) recovery percentage. Also, greatest recovery after (1.5 or 2 hrs) of dehydration with (33% or 28%) moisture contents of olive shoot tips obtained, respectively (Martinez et al., 1999). However, the reduction in the obtained survival and recovery percentage in the current study (Tables 1-4) might be attributed to partial damage of the shoot tips due to osmotic shock after rehydration and ice crystallization of some cells in the calli (Al-Ababneh et al., 2003), or might be due to unfavorable growth condition (Al-Ababneh, 2002; Moges et al., 2004). This leads to a conclusion that each plant species has its own needs for optimum dehydration to obtain best recovery after cryopreservation, and these needs must be optimized for osmotic dehydration pretreatment in terms of concentration and duration in addition to air dehydration duration.

There were other related studies in Jordan in which the cryopreservation was applied successfully on some medicinal and wild plants. For example, Sharaf et al. (2012) reported a complete survival and (27%) recovery were obtained when encapsulated non-cryopreserved *Artemisia herba-alba* shoot tips were pretreated in 0.5 M sucrose for three days. Also, Shibli et al. (2009) showed that the greatest survival (83.3; 88.9%) and recovery (77.6; 83.3%) percentages were obtained when encapsulated non-cryopreserved calluses of *Crocus hyemalis* and *Crocus moabiticus* precultured with 0.1 M sucrose for two days without dehydration, respectively. A similar result was reported by Moges, et al. (2004) who found that the full survival and the greatest recovery (75%) percentage were obtained when encapsulated non-cryopreserved African violet *Saintpaulia ionantha* shoot tips were pretreated with 0.3 M sucrose for two days with (2 hrs) air dehydration. Also, Al-Ababneh et al. (2003) reported the best survival and recovery rates in encapsulated cryopreserved bitter almond *Amygdalus communis* shoot tips air dehydrated for 6 hrs.

Table 1: Survival and recovery percentage of encapsulated shoot tips of *Ziziphora tenuior* L. as affected by air dehydration duration after pretreatment with 0.5 M sucrose concentration for one day

Sucrose Conc. (M)	Dehydration Duration (hr)*	Survival %	Recovery %	MC %
Non- cryopreserved shoots tips (-LN)				
0.5 M sucrose	0	100* ± 0.0	100* ± 0.0	85
	2	100 ± 0.0	95 ±3.49	68
	4	90 ± 4.80	75 ± 6.93	25.5
	6	80 ± 6.40	70 ± 7.33	21
Cryopreserved shoots tips (+LN)				
0.5 M sucrose	0	0.0 ± 0.0	0.0 ± 0.0	85
	2	15 ± 5.71	0.0 ± 0.0	68
	4	30 ± 7.33	20 ± 6.40	25.5
	6	35 ± 7.63	15 ± 5.71	21

*Values represent means ± standard error of mean.

Table 2: Survival and recovery percentage of encapsulated shoot tips of *Ziziphora tenuior* L. as affected by dehydration duration after pretreatment with 0.75 M sucrose concentration for one day

Sucrose Conc. (M)	Dehydration Duration (hr)*	Survival %	Recovery %	MC %
Non- cryopreserved shoots tips (-LN)				
0.75M sucrose	0	100 *± 0.0	100* ± 0.0	80.20
	2	95 ± 3.49	90 ± 4.80	51.10
	4	80 ± 6.40	65± 7.63	23.00
	6	75 ± 6.93	60 ± 7.84	19.00
Cryopreserved shoots tips (+LN)				
0.75M sucrose	0	0.0 ± 0.0	0.0 ± 0.0	80.20
	2	15 ± 5.71	0.0 ± 0.0	51.10
	4	30 ± 7.33	10 ± 4.80	23.00
	6	40.0 ± 7.84	20 ± 6.40	19.00

*Values represent means ± standard error of mean.

Table 3: Survival and recovery percentage of encapsulated shoot tips of *Ziziphora tenuior* L. as affected by dehydration duration after pretreatment with 0.5M sucrose concentration for three days

Sucrose Conc. (M)	Dehydration Duration (hr)*	Survival %	Recovery %	MC %
Non- cryopreserved shoots tips (-LN)				
0.5 M sucrose	0	100 *± 0.00	100* ± 0.00	75.8
	2	100 ± 0.00	100 ± 0.00	61.5
	4	90 ± 4.80	85 ± 5.71	26.8
	6	80 ± 6.40	70 ± 7.33	20
Cryopreserved shoots tips (+LN)				
0.5 M sucrose	0	0.0 ± 0.00	0.0 ± 0.00	75.8
	2	20 ± 6.40	0.0 ± 0.00	61.5
	4	30 ± 7.33	0 ± 0.00	26.8
	6	35 ± 7.63	10 ± 4.80	20

*Values represent means ± standard error of mean.

Table: 4: Survival and recovery percentage of encapsulated shoot tips of *Ziziphora tenuior* L. as affected by dehydration duration after pretreatment with 0.75M sucrose concentration for three days

Sucrose Conc. (M)	Dehydration Duration (hrs)	Survival %	Recovery %	MC %
Non- cryopreserved shoots tips (-LN)				
0.75M sucrose	0	100 *± 0.00	100* ± 0.00	70.30
	2	95 ± 3.49	90 ±4.80	55.90
	4	75 ± 6.93	50 ±8.00	18.10
	6	70 ± 7.33	42.5 ± 7.91	17.80
Cryopreserved shoots tips (+LN)				
0.75M sucrose	0	0.0 ± 0.00	0.0 ± 0.00	70.30
	2	0.0 ± 0.00	0.0 ± 0.00	55.90
	4	20 ± 6.40	0.0 ± 0.00	18.10
	6	10 ± 4.80	0.0 ± 0.00	17.80

*Values represent means ± standard error of mean.

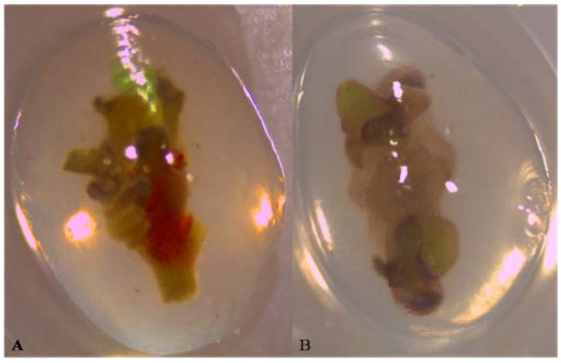

Figure 1: Survival and recovery of the cryopreserved encapsulated shoot tips of *Ziziphora tenuior* pretreated with 0.75 M sucrose MS supplemented media for one day followed by 6 hrs. air dehydration under the laminar air-flow cabinet. A: Survival, B: Recovery

3.2. Encapsulation-vitrification technique

Recovery percentages varied the encapsulated-vitrified shoot tips either with or without (LN). The greatest recovery rates (82.5%, 75%) were recorded in none cryopreserved shoot exposed to the loading solution for either (40 or 60 min) compared to (30%) recovery obtained in unloaded shoot tips (0 min) as shown in the (Figure 2). Younes (2012) reported that the shoot tips of non-cryopreserved *Achillea fragrantissima* gave the greatest survival and recovery percentages when encapsulated shoot tips were pretreated with the loading solution for 80 min. Dumet *et al.* (1993) reported that the increase in survival and recovery percentages of the non-cryopreserved shoot tips in oil palm was due to reduced osmotic shock as shoot tips were subjected to loading solution for longer duration before exposure to PVS2.

Moreover, after the cryopreservation of *Ziziphora tenuior* L. shoot tips, the greatest survival rate (37.5%) was obtained in (60 min) loading duration treatment, while shoot tips in (0, 10 min) duration treatments were not able to record survival (Figures 2- 3). Also, after cryopreservation applied to shoot tips of *Ziziphora tenuior* L. the cryopreserved shoots failed to recover in most treatments except in (40, 60 min) as (2.5, 10%) recovery rates were recorded, respectively (Figures 2- 3). However, the results obtained for recovery were very low as the highest recovery recorded was only (10%) (Figure 3) which could be attributed to intracellular ice crystals formation during freezing and or which is considered highly harmful on explants (Vandenbussche and Proft, 1996).

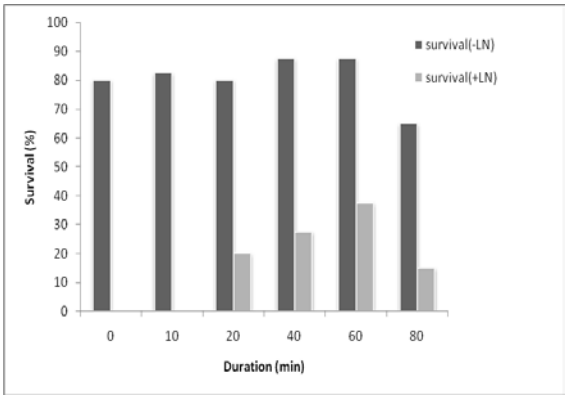

Figure 2: Survival percentages of non-cryopreserved (-LN) and cryopreserved (+LN) of *Ziziphora tenuior* L. shoot tips as influenced by loading duration with the loading solution using Encapsulation-Vitrification technique. Values represent means ± standard error of mean

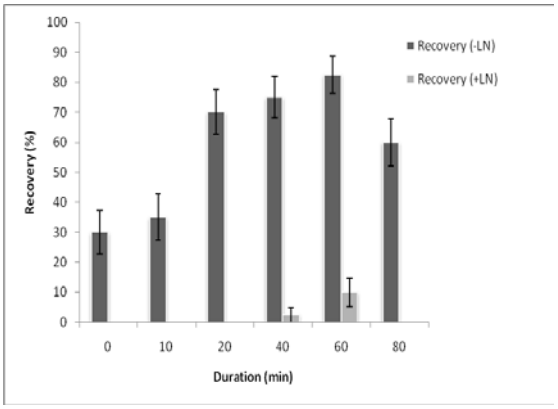

Figure 3: Recovery percentages of non-cryopreserved (-LN) and cryopreserved (+LN) of *Ziziphora tenuior* L. shoot tips as influenced by loading duration with the loading solution using Encapsulation-Vitrification technique. Values represent means ± standard error of mean

3.3. Genetic Stability

For genetic stability study on *Ziziphora tenuior* L., tissues cryopreserved and non-cryopreserved tissues (Figures 4-5) showed no clear differences when studied by AFLP. This was expected due to the fact that organized tissues (shoot tips) were used in the current study, and only undifferentiated tissues like callus and cell suspension cultures were reported to show some epigenetic variation after exposure to LN (Harding and Benson, 2001). Our results agreed with the previous studies, for example. Castillo *et al.* (2010) found no morphological differences between cryopreserved greenhouse-grown *Rubus grabowskii* when compared with the control mother plants. Lurswijidjarus and Thammasiri (2004) reported that orchid plantlets cryopreserved showed normal growth characteristics after

regrowth. Also, no variations were obtained in the morphology or growth profiles between cryopreserved and non-cryopreserved cell cultures of tobacco (Kobayashi et al., 2006). Studies of Harding (1991) and Harding and Benson (2001) on cryopreserved shoot tips of (*Solanum tuberosum* L.) confirmed that the stability of the ribosomal RNA genes and the nuclear-chloroplast DNA and the plants exhibited normal developmental patterns after re growth.

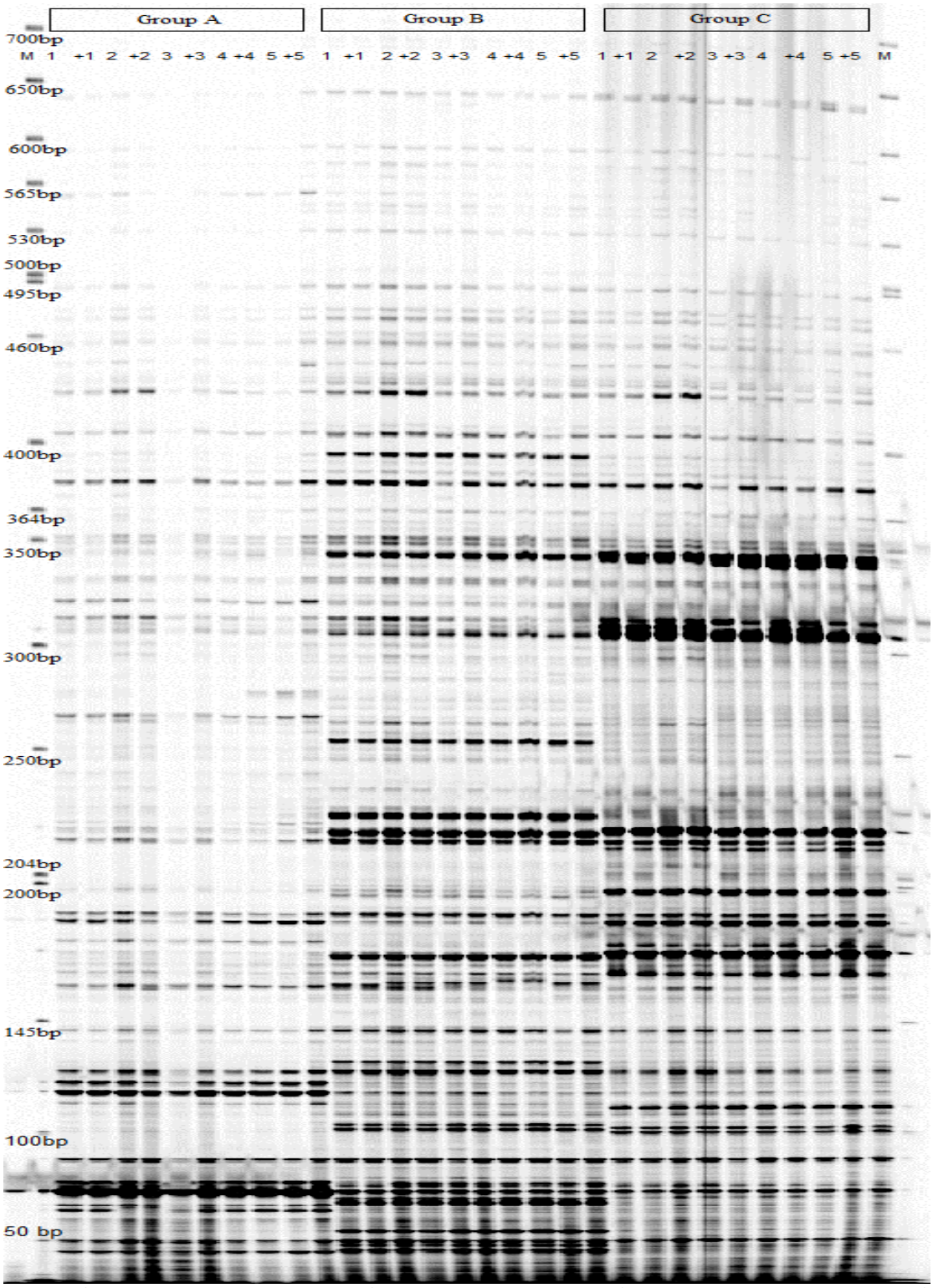

Figure 4: Amplified DNA patterns using AFLP primers for *Ziziphora tenuior* L., Pre-amplified DNA products were selectively amplified using three *Eco*RI+*MseI* primer combinations (E-ACC 700 with M-CTC (group A), M-CTA (group B), and M-CAG (group C). **(+)** stands for cryopreserved samples. Numbers from 1-5 represent cryopreserved samples of plants, respectively**.**

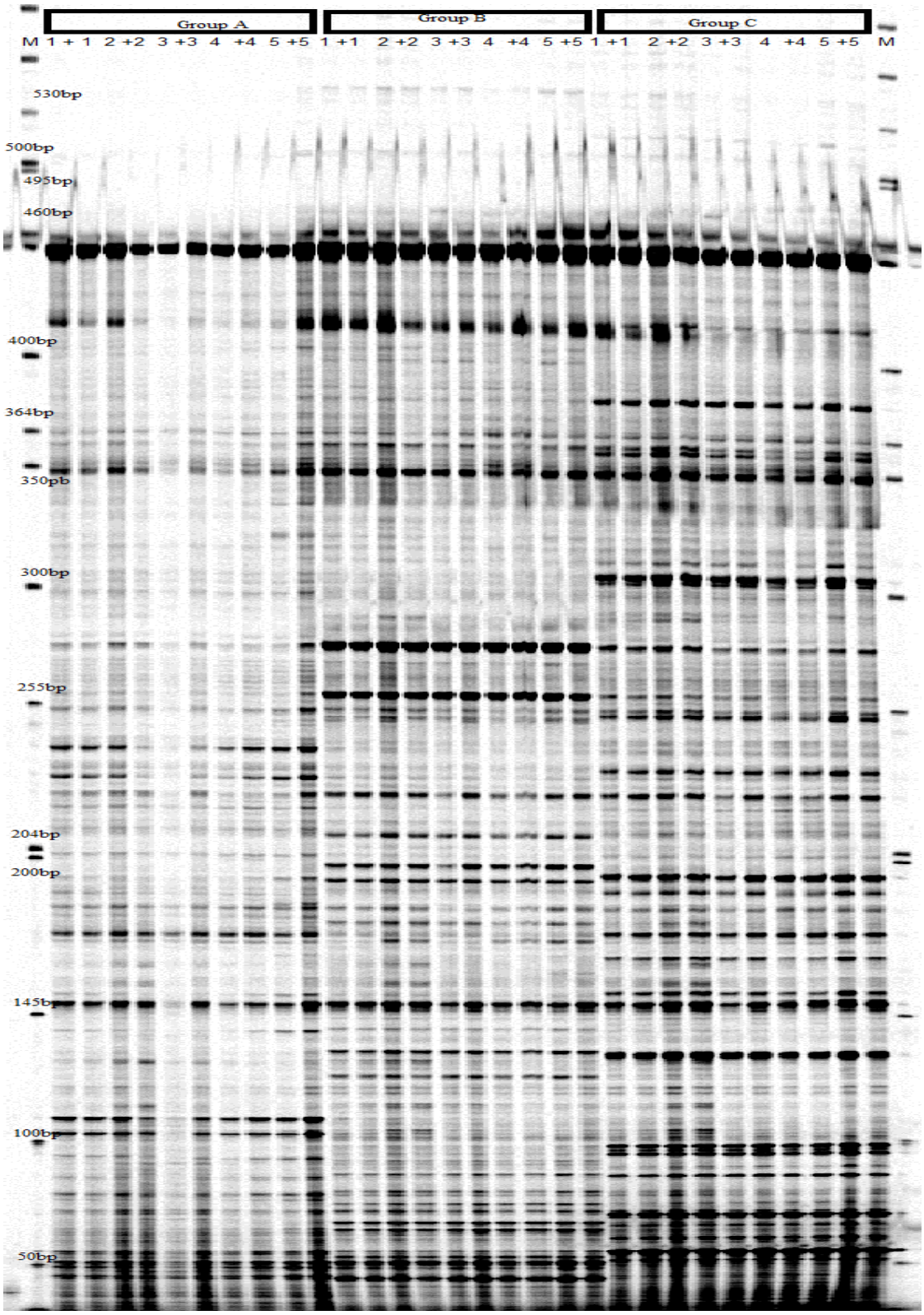

Figure 5. Amplified DNA patterns using AFLP primers for *Ziziphora tenuior* L., Pre-amplified DNA products were selectively amplified using three *Eco*RI+ *MseI* primer combinations (E-ACC 800 with M-CTC (group A), M-CTA (group B), and M-CAG (group C). (+) stands for cryopreserved samples. Numbers from 1-5 represent cryopreserved samples of plants, respectively

4. Conclusions

Encapsulation-dehydration in addition to encapsulation-vitrification methods were used for cryopreservation of *Ziziphora tenuior* L. shoot tips. In the encapsulation-dehydration experiment, the best recovery rate (20%) was obtained when either 0.5 M or 0.75 M sucrose MS supplemented media were used as preculture media for the shoot tips for one day, followed by air dehydration for 4 or 6 hrs. Meanwhile, when encapsulation-vitrification experiment was applied, the highest recovery (10%) percentage was obtained when the encapsulated shoot tips were pretreated for 60 min. with the loading solution before exposed to PVS2 vitrification solution and LN. Also, as it was expected, no genetic differences were found in the cryopreserved explants before and after exposure to LN. However, further studies on the in vitro conservation should be initiated to improve recovery rates after cryopreservation.

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Production and Characterization of a Recombinant Camel Full Heavy Chain Antibody against Human IgE

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Abstract

Camel heavy chain antibodies (HCAbs) have novel properties that render them useful in diagnosis and immunotherapy of various diseases. The purpose of this study was to produce recombinant camel HCAbs directed against a synthetic loop polypeptide that mimics the FcεRI binding site on human IgE. A recombinant camel HCAb was purified and characterized after being cloned using One Shot TOP10 *Escherichia coli*, expressed in BL21 Star (DE3) *E. coli*. Out of nineteen successful clones only one named IgG211 was found to contain the IgG2 HCAb coding sequence in the correct orientation with 85% homology to camel IgG2. A 62kDa fusion protein was expressed in an insoluble form under Isopropyl β-D-1-Thiogalactopyranoside (IPTG) induction. Probond purified fusion protein was localized by immunoblot using mouse anti camel antibody. The reactivity of recombinant camel IgG211to its corresponding antigen using ELISA was 72.9% of the camel polyclonal IgG2. Thus, a successful production of a recombinant camel HCAb of the IgG2 isotype was achieved. Such achievement may contribute towards the application of the unique properties of camel HCAbs in the field of antibody-based therapy for the treatment of asthma and allergy.

Keywords: Camels, Antibodies, Immunoglobulin E, *Escherichia coli*, DNA, Recombinant protein, Antibody-based therapy.

1. Introduction

Among mammals, the Arabian camel (Camelus dromedarius) and other Camelidae produce special kinds of antibodies in their sera, known as heavy chain antibodies (HCAbs) (Hamers-Casterman et al., 1993, Arbabi Ghahroudi et al., 1997). The HCAb molecule lacks both light chains and consists of only two heavy chains that lack the CH1 domain. The small size, high affinity, solubility, close homology of the variable domain to that of human IgG, and stability at high temperature or in the presence of denaturing agents are remarkable characteristic features for the HCAbs variable heavy chain domains (VHH). Such novel properties of HCAbs has led to intensive research towards the production of HCAbs and VHH nanobodies against various viral, bacterial, protozoal and helminthic parasites, toxins and tumors as well as other immunologic and functional protein targets. In this regard, recombinant HCAbs and VHH were successfully prepared in bacteria and yeast through cloning, expression and selections of antigen-specific HCAbs and VHHs (Van de Laar et al., 2007; Muyldermans et al., 2009; Franco et al., 2010).

IgE antibodies are reaginic antibodies involved in atopic diseases such as allergic asthmas (Beeh et al., 2000; MacLean and Eidelman, 2001; Novak and Bieber, 2003). IgE binds to its specific FcεR1 receptor mast cells as well as blood basophils. Such interaction triggers a series of cascade reactions in these cells that results in various symptoms characterizing asthma and other IgE mediated allergic diseases (Gould and Sutton, 2008).

Allergic asthma is one of the most common and highly variable chronic disorders of respiratory airways with many symptoms and complications. Prevalence of allergic asthma with severe manifestations is increasing worldwide, particularly in industrial countries (Beasley et al., 2000). The currently available treatment strategies are inadequate to control the symptoms of severe asthma (Bukstein et al., 2005). Alternative drugs, based on the production of anti-human IgE monoclonal antibodies (anti huIgE MCA), have been attempted. Such anti huIgE MCA efficiently bind IgE and do not induce histamine release in vitro (Davis et al., 1993, Presta et al., 1994, Rabe et al., 1998). Also, camel polyclonal HCAbs that

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* **List of non-standard abbreviations:** huIgE: human IgE, MCAs: monoclonal antibodies, SLP: synthetic loop peptide, IB: Immunoblot.

efficiently block IgE binding to its FcεRI on human basophils in vitro and resulted in histamine release inhibition were efficiently developed (Al-Qaoud et al., 2015). Moreover, a novel strategy based on the generation of humanized MCAs against huIgE-FcεRI binding site have been engineered and approved by the Food and Drug Administration (FDA) in the USA as early as 2003 to prevent subsequent allergic symptoms (Doding et al., 2005).

As a further approach towards the search for therapeutic strategies in asthmatic patients, an attempt to develop and purify recombinant camel HCAbs against a synthetic polypeptide that mimic FcεRI binding site on huIgE was made in the present investigation.

2. Materials and Methods

2.1. Immunogen Preparation, Peptide Synthesis and Camel Immunization

Immunogen used in camel immunization was modified Synthetic Loop Peptide (SLP) with the basic sequence CGETYQSRVTHPHLPRALMRSTTKC (Wang et al., 2003). The SLP was prepared according to multiple antigenic peptides system (MAPS) (Tam 1988) forming SLP-MAPS immunogen (Alpha Diagnostic International Co., San Antonio, USA). A Local male camel (Camelusdromedarius) was immunized with an initial 0.5 mg of SLP-MAPS mixed with Stimune adjuvant (Prionics, Schlieren-Zurich, Switzerland) at a 1:1 ratio. The camel was immunized 5 times at 2 week intervals as in our earlier work (Al-Qaoud et al., 2015). At the end of the immunization protocol, anticoagulated blood was collected for lymphocytes isolation.

2.2. Lymphocytes Isolation, RNA Extraction and cDNA Synthesis

Lymphocytes were obtained from heparinized peripheral blood samples of SLP-MAPS immunized camel using Ficoll-Paque (PAA laboratories, Linz, Austria). The cell number was adjusted to (5×106) for RNA isolation. RNA was isolated using a Nucleospin RNA II Total RNA isolation kit (Macherey-Nagel, Düren, Germany) following the manufacturer's instructions. Fiveμgof RNA was converted to cDNA by Reverse Transcriptase kit (Promega, Wisconsin, USA). The cDNA was used as a template to amplify the coding sequence for camel HCAbs.

2.3. Design of PCR Primers and pET102/D-TOPO Expression Vector Cloning Site

Two separate PCRs were done to clone the HCAb coding sequence in the pET102/D-TOPO expression vector using a shared reverse primer (Cam2) and one of two different forward primers. The forward primers that were used in the first and second PCRs were called CamV1 and CamV-Topo, respectively. The sequences of the primers were as follows:
CamV1: (5′ ACACACCATGGAGCTGGGGC 3'),
CamV-Topo:(5′ CACCGATGTGCAGCTGCAGGAGTC 3′), and
Cam2:(5'CTAGTGGGTCAGAAGCCCATTT3')
(Saerens et al., 2004).

The pET102/D-TOPO expression vector was designed to directionally clone the gene between N-terminal HP-thioredoxin and C-terminal V5 epitope and 6xHis tags and allows the inducible expression of proteins in *E. coli* strains under the control of T7 promoter (Figure 1).

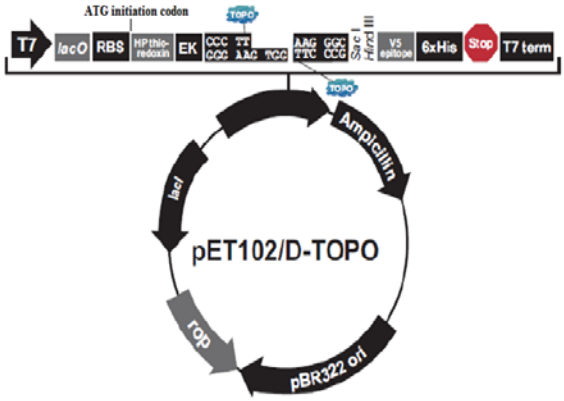

Figure 1: The pET102/D-TOPO expression vector cloning site and characteristics. The vector contains T7 promoter, lac operator (lacO), Ribosome binding site (RBS), ATG initiation codon, His-patch (HP) thioredoxin open reading frame (ORF), TrxFus forward priming site, EK recognition site, directional TOPO Cloning site, V5 epitope, Polyhistidine (6xHis), T7 reverse priming site, T7 transcription termination region, Ampicillin resistance gene, and pBR322 origin of replication.

2.4. Production of Blunt-End PCR Products

The HCAbcoding sequence was amplified by nested PCR using Phusion HF DNA polymerase(New England BioLabs, Massachusetts, USA) and ESCO Swift Max Pro Thermal Cycler (Esco Technologies, Changi, Singapore).The first PCR was done to separate HCAbs from the conventional IgG1. The PCR was done with 35 cycles at 60C annealing temperature. Separation of PCR product was done using 1% agarose gel electrophoresis after staining with ethidium bromide. The two bands near 1200 and 1300 base pairs, representing the two HCAbs isotypes coding sequences, were used as templates for the secondary PCR. The HCAbs were amplified in the second PCR reaction and the bands representing IgG2 and IgG3 coding sequences from the two second PCR reactions were extracted using Wizard SV Gel and PCR Clean-up system kit (Promega, Wisconsin, USA).

2.5. Assembly of the HCAb Expression Construct and Transformation of One Shot TOP10 E. coli Competent Cells

The Champion pET Directional TOPO Expression Kit (Invitrogen, California, USA) was used for cloning. The purified IgG3 and IgG2 PCR products were cloned into pET102/D-TOPO vector as per manufacturer instructions. The ligation mixture was transformed into One Shot TOP10 *E. coli* using the heat shock method.

2.6. Analysis of Positive Transformants by Colony PCR and DNA Sequencing

All the colonies that appeared on the LB-Amp (100μg/ml) plates were prepared for analysis by colony PCR using the TrxFus forward and T7 reverse sequencing primers supplied with the Topo cloning kit. The positive construct was sequenced to check the identity and orientation of the HCAb coding sequence using the

TrxFus forward sequencing primer (5'-TTCCTCGACGCTAACCTG-3´) with the aid of Eaton Corporation (Ohio, USA).The pET102/D-TOPO-HCAb construct was extracted using GenElute plasmid miniprep kit (Sigma, Missouri, USA) following specific manufacturer instructions.

2.7. Expression of the Recombinant HCAb Fusion Protein

Seven ng of the pET102/D-TOPO containing the expected anti-SLP-MAPS HCAb sequence was transformed into BL21 Star (DE3) *E. coli* using heat shock method following themanufacturer instructions. The culture containing the transformation reaction was grown overnight at 37ºC with shaking at 200 rpm and induced with 1mM Isopropyl β-D-1-thiogalactopyranoside (IPTG) (Promega, Wisconsin, USA). The cells were harvested and resuspended in lysis buffer (50 mM potassium phosphate, 400 mM NaCl, 100 mM KCl, 10% glycerol, 0.5% Triton X-100, 10 mM imidazole, pH 7.8). The cells were lysed by freezing/thawing cycles (five times) using liquid nitrogen and 42°C water bath. After centrifugation, the supernatants containing the soluble fraction and pellets which contained the insoluble fraction were both used for analysis on 12% SDS-PAGE.

2.8. Purification of the Recombinant HCAb Fusion Protein and Analysis by SDS-PAGE

A fifty ml-sample of IPTG induced culture was used for the purification of the recombinant camel HCAb fusion protein using ProBond Purification System (Invitrogen, California, USA). Probond column was washed seven times and the unbound fractions were removed as per manufacturer instructions. Fractions were collected and analyzed by 12% SDS-PAGE.

2.9. Assessment of Recombinant Fusion HCAb by ELISA and Immunoblot (IB)

ELISA was used for the detection of the binding activity of the recombinant HCAb fusion protein with the SLP-MAPS synthetic peptide. Captured camel anti SLP-MAPS HCAbs were detected by HRP-labeled mouse anti camel antibodies prepared according to MonoJo's Standard Operating Procedures (SOP). Furthermore, the purified recombinant HCAb fusion protein, the induced bacterial lysate and uninduced bacterial culture were electrophoresed by SDS-PAGE and blotted on a nitrocellulose membrane using semidry immunoblotter purchased from Wealtec (Taipei, Taiwan). The presence of camel HCAbs was detected by the incubation with mouse anti camel-HRP as tracer. A relative reactivity index for recombinant IgG211 to anti-huIgE polyclonal IgG2 was calculated as per the formula: the reactivity of recombinant IgG211 divided by the reactivity of polyclonal IgG2 multiplied by 100.

3. Results

3.1. Camel HCAb Gene Amplification and Transformation

Nested PCR was performed to amplify the HCAbs coding sequence from lymphocytes of SLP-MAPS immunized camel (Figure 2). The resulting PCR products from the first reaction were three bands near 1500, 1300, and 1200bp. These bands represent camel IgG1, IgG2 and IgG3 coding sequences, respectively (Figure 2a). The second PCR re-amplified the gel purified IgG2 and IgG3 coding sequences (Figure 2b).The constructs containing the gel purified IgG2 and IgG3 coding sequences were transformed into TOP10 *E. coli* competent cells. The ligation efficiency was very low where only 15 and 4 bacterial colonies that resultedfrom the transformation of constructs contained IgG2 and IgG3 coding sequence, respectively.

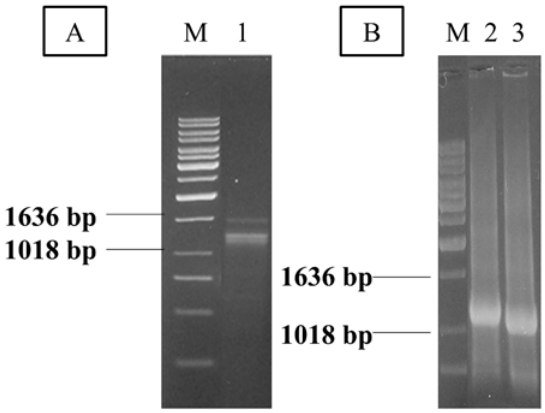

Figure 2: Nested PCR amplification products using 1% agarose gel. CamV-1 and Cam2 primers were used on camel lymphocyte cDNA.In A, IgG1 appears as a discrete band of ~ 1500bp while the two bands near 1018bp represent the HCAbs IgG2 and IgG3 complete coding sequence (Lane1). In B,CamVTopo and Cam2 primers were used on the gel purified IgG2 coding sequence (Lane 2), and on the gel purified IgG3 coding sequence (Lane 3), M: 1Kb DNA ladder.

3.2. Colony PCR and DNA Sequencing of Positive Transformants

All the 4 and 15 colonies were analyzed by colony PCR using the TrxFus forward and T7 reverse primers that annealed on the pET102 vector around the cloning site. Accordingly, the expected size of the clone containing the plasmid with an insert was around 1500bp. None of the 4 colonies yielded positive result, whereas only colony number 11 from the 15 colonies that resulted from transforming construct containing IgG2 coding sequence yielded positive result (Figure 3). This clone was named IgG211. Alignment of the sequence result of IgG211 on the NCBI database revealed homology with *Camelus dromedarius* heavy chain region with 84% identity. The sequence alignment showed a plus/plus result which indicates that the gene is in correct orientation.

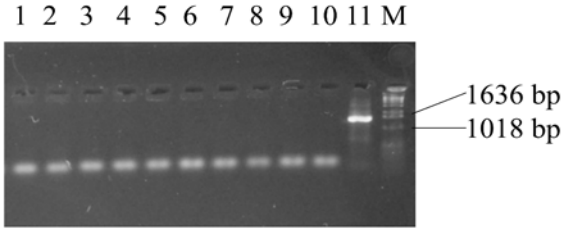

Figure 3: Colony PCR analysis of bacterial transformants harboring the pET102/D-TOPO-IgG2 construct using the TrxFus Forward and T7 Reverse primers. (Lanes 1-11) are bacterial colonies. Only colony number 11 contained the construct with the IgG2 coding sequence that was named $IgG_2$11. M: 1Kb DNA marker.

3.3. Expression, Analysis and Purification of Recombinant Fusion IgG211

Both the IPTG induced and uninducedIgG211 in BL21 (DE3) *E. coli* were both analyzed using SDS-PAGE to test for the expression of IgG2. Evidently, an over-expressed band near 65 kDawas revealed in the induced but not in the un-induced culture (Figure 4). Meanwhile, the expected band size was approximately 62 kDa including the expression of the N-terminal HP-thioredoxin and C-terminal V5 and 6His epitopes. The IPTG induced bacterial culture was analyzed for soluble/insoluble protein. A fusion form of IgG211 protein of approximately 65 kDa was completely found in the insoluble fraction (Fig 4). The recombinant IgG211 fusion protein was eluted and most of it was eluted in the first two fractions that yielded approximately 70% of the protein (Figure5).

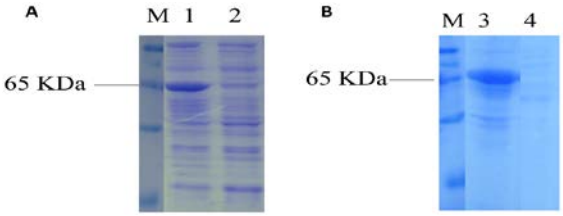

Figure 4: SDS-PAGE of IPTG induced and un-induced BL21 (DE3) *E coli* cell lysate harboring the $IgG_2$11 construct (A). Lane 1 for induced and lane 2 for un-induced. In B induced lysate was analyzed for insoluble (lane 3) and soluble protein (Lane 4). IgG211 appeared completely in the insoluble fraction (B lane 3). M: prestained molecular weight marker.

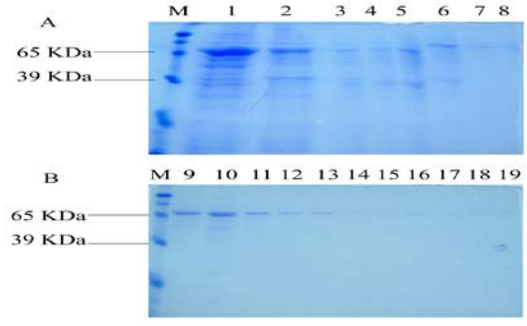

Figure 5. Purification of IgG211 from a 50 mL of induced BL21 bacterial culture using ProBond column. A 12% SDS-PAGE was done on washing (A) and elution (B) fractions. (Lane 1): induced BL21 bacterial cell lysate with abundant IgG211 fusion protein; (Lanes 2-8): wash fractions, (Lanes 9-19) IgG211 elution fractions with native elution buffer pH 7.8.

3.4. Activity Assessment of Purified Recombinant IgG211 Fusion Protein

The purified recombinant IgG211 fusion protein was analyzed by IB using mouse anti camel-HRP to confirm its identity. Purified IgG211 was captured by the mouse anti camel-HRP and appeared as discrete faint band. In addition, many nonspecific bands appeared in the un-induced bacterial lysate (Figure 6). Furthermore, the specificity of the purified recombinant IgG211 fusion protein was analyzed by ELISA. Low binding specificity was observed using SLP-MAPS as coating antigen. However, the relative reactivity index of recombinant IgG211 compared to polyclonal IgG2 was65.9% (Figure 7).

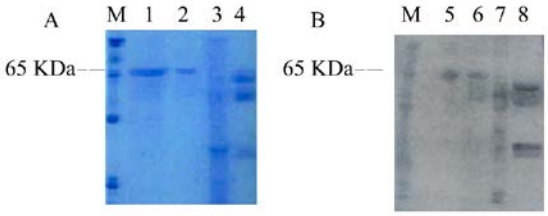

Figure 6. SDS-PAGE (A) and IB with mouse anti camel-HRP as a tracer (B) of the purified $IgG_2$11. (Lanes 1 and 5): induced BL21 bacterial lysate; (Lanes 2 and 6): purified $IgG_2$11; (Lanes 3 and 7): un-induced BL21 bacterial lysate; (Lanes 4 and 8): a mix of purified camel IgG1 and IgG3 as positive control, respectively.

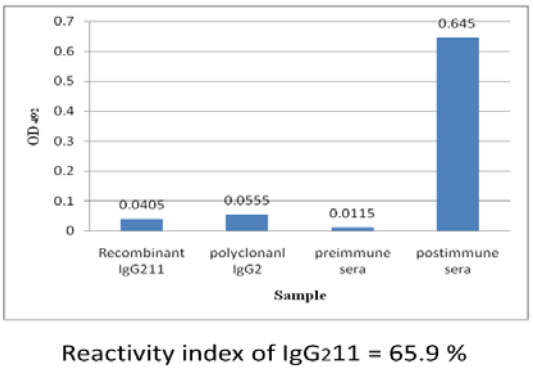

Figure 7. ELISA results of purified recombinant $IgG_2$11 to SLP-MAPS. Both preimmunized and postimmunized camel sera were used as negative and positive controls, respectively.

4. Discussion

As research on the dissection of camel immune response and antibody production is still in early stages, the lack of fusion myeloma partner for antibody production hindered the production of camel monoclonal antibodies (MCAbs). Nevertheless, recombinant HCAbs and VHH were successfully prepared through cloning, expression and selections of antigen-specific HCAbs and VHHs in bacteria and yeast (Arbabi Ghahroudi et al., 1997; Van der Vaart et al., 2006; Muyldermans et al., 2009; Franco et al., 2010). In this study, we produced a recombinant full camel HCAb against human IgE in *E coli* for the first time. IgG2 HCAb with coding sequence

in the correct orientation was confirmed upon the cloning and transformation in one *E. coli* colony named IgG211.

The IPTG-inducible expression of the recombinant IgG211 fusion protein in BL21 (DE3) *E coli* revealed that the IgG211 fusion protein was completely found in the insoluble fraction. This was opposite to what was expected, due to the presence of HP-thioredoxin at the N-terminal end which enhances solubility. However, the possible formation of insoluble recombinant proteins in form of inclusion bodies using *E coli* as n expression host was previously described (Ventura and Villaverde, 2006). Those workers indicated that aggregation of protein in the inclusion bodies renders it as unfolded or improperly folded protein.

The 46kDa size IgG211 was eluted as part of the 62kDa fusion protein which contained a 16 kDa HP-thioredoxin at the N-terminal end and a 6His-tag and V5 epitope at the C-terminal end. Due to the presence of the 6His-tag at the C-terminal of the IgG211 fusion protein, it was easily purified using ProBond nickel-chelating resin. Over 70% IgG211 was recovered in the first two elution fractions with relatively high purity. This high purity can be related to its presence in the inclusion bodies aggregates that is common to occur in *E coli* expression host (Ventura and Villaverde, 2006).

The other observation pertaining to this purified recombinant IgG211 fusion protein was its low reactivity against SLP-MAPS immunogen using the ELISA (Fig. 7). In our earlier work, (Al-Qaoud et al., 2015), we showed that the reactivity polyclonal IgG2 against SLP-MAPS was low using the ELISA, but was highly reactive using the flow cytometry technique. Thus, further analysis of the IgG211 recombinant protein by flow cytometry is warranted.

The production and use of orally administered anti huIgE HCAbs as immunotherapeutic agents is of great importance. This is due to several reasons which prefers the use of the aforementioned HCAbs compared to conventional antibodies. The high stability, solubility, affinity and most importantly low immunogenicity of HCAbs has been repeatedly documented. In addition, no cytotoxicity or anti-nanobody antibodies were detected in serum of mice injected with nanobodies for therapeutic purposes (Dumoulin et al., 2002; Cortez-Retamozo et al., 2004; Revets et al., 2005; Coppieters et al., 2006) that favors camel nanobodiesto be used in the treatment of various diseases. Moreover, high sequence homology between camel VH and human VH was confirmed by amino acid sequence analysis that revealed a difference of only 14 amino acid which in turns potentiate humanization process and rendering the humanized camel HCAb less immunogenic than humanized conventional antibody (Vincke et al., 2009; Deschacht et al., 2010). Furthermore, one key advantage that favor the use of camel HCAbs as blocking non-anaphylactogenic anti huIgE antibodies is their monovalent nature in contrast to the bivalent nature of mouse conventional IgG allowing HCAbs capable of targeting free IgE but not crosslinking bound IgE (Harmsen and De Haard, 2007). With these novel properties it is expected that the use of camel HCAbs as anti huIgE blocking antibodies will overcome the side effect that accompanied the use of the commercially available humanized mouse anti huIgE known as Omalizumab (Davydov, 2005; Doding et al., 2005; Slavin et al., 2009).

5. Conclusions

In conclusion, the superiority of camel HCAb (IgG2 and IgG3) against huIgE Cε3 over conventional camel IgG1 as revealed in earlier work (Al-Qaoud et al., 2015) paved the way toward successful production of recombinant camel HCAbs against SLP-MAPS. IgG211 was produced and expressed in E. coliand initial experiments to characterize it were done here. Evidently, the selection of higher affinity HCAb may be achieved by other techniques such as phage display technology which offers larger repertoire of antigen specific antibodies (Carmen and Jermutus, 2002; Arbabi-Ghahroudi et al., 2009). Presently, we are trying to produce camel nanobodies to be used as orally administered therapeutic drug against huIgE using the recombinant phage display technology.

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EntomoToxicity of *Xylopia aethiopica* and *Aframomum melegueta* in Suppressing Oviposition and Adult Emergence of *Callasobruchus maculatus* (Fabricus) (Coleoptera: Chrysomelidae) Infesting Stored Cowpea Seeds

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Abstract

The cowpea beetle, *Callosobruchus maculatus* (Fabricus) (Coleoptera: Chrysomelidae), is a major pest of stored cowpea militating against food security in developing nations. The comparative study of *Xylopia aethiopica* and *Aframomum melegueta* powder in respect to their phytochemical and insecticidal properties against *C. maculatus* was carried out using a Complete Randomized Design (CRD) with five treatments (0, 1.0, 1.5, 2.0 and 2.5g/20g cowpea seeds corresponding to 0.0, 0.05, 0.075, 0.1 and 0.13% v/w) replicated thrice under ambient laboratory condition (28±2°C temperature and 75±5% relative humidity). The phytochemical screening showed the presence of flavonoids, saponins, tannins, cardiac glycoside in both plants, while alkaloids was present in *A. melegueta* and absent in *X. aethiopica*. The mortality of *C. maculatus* increased gradually with exposure time and dosage of the plant powders. *X. aethiopica* caused 75.15% adult mortality and *A. meleguta* exerted 85% mortality at 120 hrs post infestation. Maximum oviposition deterrent activity was observed with *X. aethiopica* (54.26%) compared to *A. melegueta* (51.32%). Conclusively, both plants showed highly useful bioactivity against *C. maculatus* in suppressing oviposition and adult emergence and, therefore, can be used in formulating ecofriendly herbal insecticides.

Keywords: Adult emergence, bioactivity, herbal insecticides, insecticidal properties, oviposition deterrent, phytochemical.

1. Introduction

Cowpea (*Vigna unguiculata* L.) is an important food crop that accounts for about 60% of human dietary protein intake and can provide a comparatively cheaper alternative to animal proteins in Nigeria. The high protein, amino acid and lysine contents of the seeds make them a natural supplement to staple diet cereals, roots, tubers and fruits (Somta *et al.*, 2008). Stored cowpea grains are heavily infested by the cowpea beetle, *Callosobruchus maculatus* (Fabricus) (Coleoptera: Chrysomelidae) and caused over 90% of the insect damage to cowpea seeds. This is the cause of the main reduction in cowpea production (Radha and Susheela, 2014). Damaged cowpea seeds are unsuitable for human consumption and cannot be effectively used for agricultural and commercial purposes as result of the substantial reduction in both quantity and quality.

Management of *C. maculatus* on stored cowpea in Nigeria and developing nations has been primarily through the use of synthetic fumigants over the years. While these synthetic fumigants control are popular and effective, their improper application has resulted in environmental, human health problems and insect resistance. These serious limitation posed by the use of synthetic fumigants as preservatives during storage called for the search for new alternative methods of controlling the stored product insect pests, such as the use of promoting plant products (Ileke *et al.*, 2014). Certain

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plants possess secondary metabolites which act as antifeedants, oviposition deterrents, larvicidal and insect growth regulators (Pugazhvendan *et al*., 2009). They hold a promise as alternatives to chemical insecticides to reduce pesticide load in the environment and food chain.

Xylopia aethiopica (Dunal) A. Rich. (Family: Annonaceae), popularly known as African pepper, Ethiopian pepper or spice (Ndukwu and Ben-Nwadibia, 2005), is an important, deciduous evergreen, aromatic/medicinal plant, growing up to 20 m high and widely distributed in low land rain forest and moist fringe forests in the Savanna zones and coastal regions of Africa (Kieta *et al*., 2003). Concoctions prepared from its morphological parts are used in traditional medicine for the treatment of skin infection, candidiasis, cough, fever, dysentery and stomach ache (Okigbo *et al*., 2005). Extracts from *X. aethiopica* have been reported to exhibit mosquito repellent (Adewoyin *et al*., 2006) and termite antifeedant (Lajide *et al*., 1995) activities. The fruit powder and its essential oil were found effective to control maize weevil (*Sitophillus zeamais* Motsch) (Kouninkil *et al*., 2005).

Aframomum melegueta K. Schum., known as Alligator pepper or Grains of Paradise (Family *Zingberaceae*), is a herbaceous tropical perennial West Africa spice plant with a short stem growing up to 5m tall, highly branched with lanceolate leaves and adventitious roots (Okujagu, 2008). *A. melegueta* is a very popular spice which imparts a pungent peppery flavor with hints of citrus and used mainly as food, in brewing, and in both veterinary and traditional medicine (Igwe *et al*., 1999). It is believed to have purgative and hemostatic properties and also to be very effective against schistomiasis (Alaje *et al*., 2014). Various researchers reported the potential of *A. melegueta* for the management of stored products' insect pests (Ofuya, 1990; Adedire and Lajide, 1999; Onekutu *et al*., 2015).

However, the ovipositional deterrents activity of the plants documentation is scare and in light of the foregoing, the present study assesses the oviposition deterrent and progeny suppression potential of *X. aethiopica* and *A. melegueta* on *C. maculatus* infesting stored cowpea.

2. Materials and Methods

The experiment was conducted at the Entomology Laboratory, Department of Crop, Soil and Pest Management Technology, Rufus Giwa Polytechnic, Owo, Ondo State, Nigeria (7° 11' N and 5° 35' E) under ambient temperature of 28±2°C, 75±5% relative humidity and 12hrs photoperiod. The initial *C. maculatus* culture was obtained from an infested stock of cowpea bought from Ulede Market, Owo, Ondo State, Nigeria and sub-cultured on Sokoto white (a susceptible cultivar) in a 2 l kilner jar, covered with muslin cloth to allow for proper ventilation. The jar was kept for the insect to breed and multiply in order to supply insects for the study.

Fruits of *X. aethiopica* and *A. melegueta* were obtained from traditional herbal trader at Ulede Market, Owo, Ondo State, Nigeria and were authenticated at the Forestry and Wood Technology Department, Rufus Giwa Polytechnic, Owo, Ondo State, Nigeria. The fruits were rinsed in clean water and air dried. The seeds were made into powder using mortar and pestle. The powder was further sieved and packed into plastic containers with tight lids and stored in a laboratory cupboard until use.

Cowpea (cv. Oloyin), used for the experiment, was bought from the food grain stall at Ulede market, Owo, Ondo State, Nigeria. Damaged seeds were sorted out by handpicking leaving healthy seeds; these were nevertheless kept in a deep freezer at -15°C for 48 hrs to eliminate any stages of the insect. Powder of *X. aethiopica* and *A. melegueta* were evaluated at different concentrations of 0, 1.0, 1.5, 2.0 and 2.5g corresponding to 0.0, 0.05, 0.075, 0.1 and 0.13% v/w mixed thoroughly and separately with 20g of uninfested cowpea seeds in 125 ml plastic container. Ten (2-3 days old) unsexed *C. maculatus* adults were introduced into each plastic containers; these were covered to prevent entry and exist of insects. There was also a control treatment that did not involve the addition of any plant powder onto the seeds. Adult mortality was monitored at 24-h-interval (24, 48, 72, 96 and 120 hrs) post infestation. Insects were considered dead when they did not respond to gentle pressure using a fingertip. To avoid the possibility of death mimicry, the insects were watched for 2 min and again subjected to gentle pressure. Adult mortality was corrected using abbot's formula (Abbot, 1925).

The oviposition deterrent activity was assessed by admixing 0, 1.0, 1.5, 2.0 and 2.5g corresponding to 0.0, 0.05, 0.075, 0.1 and 0.13% v/w of *X. aethiopica* and *A. melegueta* powder with 20g of uninfested cowpea seeds in 125 ml plastic containers. Ten (2-3 days old) unsexed *C. maculatus* adults were introduced into each plastic container covered with lid. The insects were allowed to remain in the container for 7 days to allow the insect oviposit. The number of eggs laid on treated and control containers were counted using a hand lens and the percentage of oviposition deterrent activity was calculated using the formula adopted by Arivoli and Tennyson (2013).

% oviposition deterrent activity =

$$\frac{\text{no of eggs laid in control dish} - \text{no of eggs laid in treated dish}}{\text{no of eggs laid in control dish} + \text{no of eggs laid in treated dish}} \times \frac{100}{1}$$

To determine the F1 progeny deterrent efficacy of the plant powder, 20g of cowpea seeds were placed in 125 ml plastic container and admixed with different dosage rates of *X. aethiopica* and *A. melegueta* powder as stated above. After the egg count, the experimental set up was kept undisturbed until the emergence of F1 adults. The number of F1 adults that emerged from each replicate in the control and treated seeds were counted with the aid of aspirator and recorded at 30 days post infestation and was used to calculate percentage reduction in adult emergence.

% reduction in adult emergence =

$$\frac{\text{no of emerged adult from control dish} - \text{no of emerged adult from treated dish}}{\text{no of emerged adult from control dish}} \times \frac{100}{1}$$

The phytochemical screening of the plant powders for tannins, saponins, alkaloids and cardiac glycosides were carried out by the methods described by Harborne (1973), Sofowora (1993) and Trease and Evans (1998). For tannin, 5 g of each portion of plant powder was stirred with 10 ml of distilled water and filtered as described by

Trease and Evans (1998). Blue black, green, or blue-green precipitate formed following the addition of few drops of 5% ferric chloride confirmed the presence of tannins.

Salkowski's test, as described by Sofowora (1993), was used to test cardiac glycosides. Plant powder (0.5 g) was dissolved in 2 ml of chloroform prior to the careful addition of 1% (v/v) H_2SO_4 to form a lower layer. A reddish-brown colour at the interface confirmed that cardiac glycoside was present.

In Alkaloids determination, 0.5g of each powder was stirred with 5ml of 5% aqueous HCl on water bath and filtered; 1ml of the filtrate was treated with a few drops of Dragendorff's reagent. Precipitation or turbidity was taken as preliminary evidence for the presence of alkaloids in the plant being evaluated (Harborne, 1973).

The method described by Ekpo *et al*. (2012) was used to determine Saponins. About 0.5g of each plant extract was shaken with 10ml of distilled water in a test tube. Frothing which persists on warming was taken as preliminary evidence for the presence of saponins.

For flavonoids, about 0.5g of each extract was stirred with few drops of Mg strips and conc. HCl was then added. A reddish coloration indicates a positive test for flavonoids (Sofowora, 1993).

Prior to analysis, egg count and adult emergence were subjected to square root transformation and percentages were arc sine transformed to normalize data. All data collected were subjected to Analysis Of Variance (ANOVA) using Statistical Package for Social Sciences (SPSS) for windows version (SPSS, 1999). Treatment means were separated using Least Significant Difference (LSD) at 5% probability level (Gomez and Gomez, 1984). The graph was designed using MS Excel 2010 version.

3. Results and Discussion

The contact toxicity on the survival of adult beetles after treatment with the plant powders is presented in Figure 1. The mean percent mortality of *C. maculatus* was observed to be directly proportional to the exposure period and concentration. Although none of the tested plant powder was able to exert 100% adult mortality, *A. melegueta* 0.13% v/w (2.5g/20g cowpea) caused 81.14% adult mortality and *X. aethiopica* exerted 70% adult mortality at 120 hrs post infestation. The striking effects of plant powders could be attributed to the presence of their toxic components and irritating smell which prevented physical contact of adult weevils with grains and caused suffocation or starvation of the pest (Sarwar *et al*., 2012) or to the induction of some unknown physiological changes (Mathur *et al*., 1985), which significantly ($P<0.05$) reduced the number of the tested insect. This shows that both powders contained toxic ingredients for *C. maculatus*. The insect mortality may equally be due to blocking of spiracles of the insect by dust particles and death caused by asphyxia (Adedire *et al*., 2011; Fernando and Karunaratne, 2012). Further, it was revealed that the plant powder may cause abrasion of insect cuticle, which led to water loss. The water loss in the insect ultimately results in its death (Sousa *et al*., 2005). The significantly high mortality rate indicates the probable presence of insecticidal properties in the plants. This confirms the findings of Ajayi and Wintoba (2006) and Onekutu *et al.* (2015) that reported the insecticidal bioactivity of the tested plant against *C. maculatus* infesting stored cowpea.

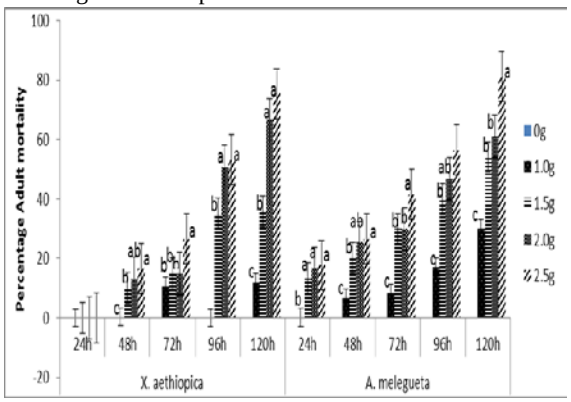

Figure 1. Corrected Mean Percentage Adult Mortality of *C. maculatus* treated with powder of *Xylopia aethiopica* and *Aframomum meleguta.* Means with the same letter for each plant powder are not significantly different using LSD at 95 % confidence level.

The data shown in Figure 2 revealed the effect of the evaluated plant powders on oviposition deterrent of *C. maculatus*. The result shows that the reduction in oviposition increases with the dosage increase. The higher dosage shows that the insects treated with 2.5g/20g cowpea seeds were found to be effective in suppressing egg laying as compared to lower dosage rate; although no concentration of the plant powders could completely prevent the females from oviposition. Maximum oviposition deterrent activity was observed with *A. melegueta*. This corroborates with the findings of Olaifa and Erhun (1998) and Adesina and Ofuya (2015) who found out that higher concentration of plant powder of *Piper guineense* and *Secamone afzelii* extracts, respectively, significantly reduced *C. maculatus* oviposition. It is noteworthy that all these plant powders showed more than 50% deterrent activity and significantly suppressed oviposition, even at lower concentration compared to unprotected cowpea seeds. Though, Ofuya (1990) reported that both seed powder and extract of *A. melegueta* did not significantly affect oviposition and egg hatchability of *C. maculatus* but, from the present study, it appears that these plant powders might possess oviposition deterrent principles; the survival and egg laying of *C. maculatus* was significantly affected by the treatment suggesting the presence of insecticidal and ovipositional active compound in the plants which delayed and completely inhibited the oviposition of the insects. This is in agreement with the findings of Abdullah and Muhammad (2004) who reported that the powder of *Piper guineense* adversely affected survival and egg laying capacity, higher ovicidal effects, reduced oviposition rates. The deterrent activity of the insect might be attributed to the change in the behavior and physiology of the insect after the treatment with the plant powders due to the chemical nature of the powder which adversely affects the egg laying capacity (Shifa Vanmathi, 2010). Besides, the plant powder can reduce insect movement, sexual communication and disrupts mating activities and as well as deterring females from laying

eggs (Ileke *et al.*, 2012). The present study is in agreement with the findings of Dolui *et al.* (2010) and Dolui *et al.* (2012) who reported a considerable reduction in the number of eggs laid per female *Helopeltis theivora* after treatment with *X. aethiopica*. In a related development, Gehlot and Singhvi (2006) and Ravinder (2011) reported that oviposition of *C. maculatus* was also significantly reduced by the treatment of Eucalyptus leaf extract, turmeric powder, black pepper powder and garlic clove powder.

It is pertinent to note that shortened adult life-span by the treatments must have also been responsible for reduced oviposition. More so, since female beetles deposit most of their eggs in the first 3 days of adult life (Wasserman, 1985), any reduction in adult lifespan, as a result of the plant powder would be expected to have, contributed to the reduced oviposition

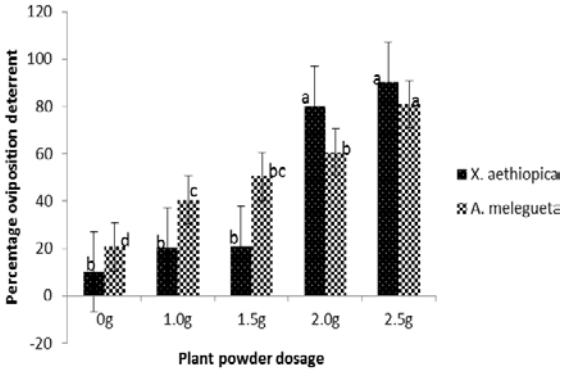

Figure 2. Mean percentage oviposition deterrent of *C. maculatus* on treated cowpea seeds. Means with the same letter for each plant powder are not significantly different using LSD at 95 % confidence level.

A significant reduction in adult emergence was recorded among the various treatments (Figure 3). In the present study, adult emergence decreased significantly ($P<0.05$) as the concentration of the powders increased. The result indicated that the adult emergence reduction is dosage rate dependent. The reduction in adult emergence could either be due to egg mortality or larval mortality or even reduction in the egg hatching. It has been reported that the larvae hatching from the eggs of *Callosobruchus* species must penetrate the seeds to survive (FAO, 1999). The plant powders might have inhibited the larval penetration into the seed and thus showed maximum adult emergence reduction (Khalequzzman and Goni, 2009). Jayakumar *et al.* (2003) reported that plant products have obvious effects on postembryonic survival of insects and resulting to reduction in adult emergence in all the concentrations of different plants. Annie Bright (2001) and Raja *et al.* (2001) reported that botanicals inhibit adult emergence of C. *maculatus* in cowpea. They further stated that when the eggs were laid on treated seeds, the toxic substance present in the plant products may enter into the egg through chorion and suppressed their embryonic development, thus, reduced adult emergence. The ability of the evaluated plants to significantly ($P<0.05$) suppress adult emergence suggested that the plants might possess ovicidal and larvicidal properties and this confirms the findings of Ofuya (1990).

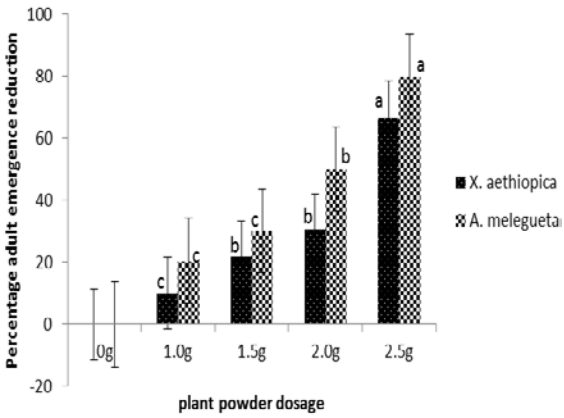

Figure 3. Mean percentage adult emergence reduction of *C. maculatus* protected with *Xylopia aethiopica* and *A*framomum *melegueta*. Means with the same letter for each plant powder are not significantly different using LSD at 95 % confidence level.

The results of the phytochemical constituents of both plant powders, as shown in Table 1, revealed the presence of tannins, cardiac glycosides and saponins in both plants, while alkaloids is absent in *X. aethiopica* and present in *A. melegueta*. Secondary metabolites such as phenolic compounds, saponins alkaloids, flavonoids and terpenoids have been identified to exhibit strong activities against several pathogens and insect pests (De Geyter *et al.*, 2007).

Table 1. Phtyochemical constituents of *Xylopia aethiopica* and *Aframomum melegueta*

Phtyochemical	X. aethiopica	A. melegueta
Alkaloids	-ve	+ve
Flavonoids	+ve	+ve
Cardiac glycosides	+ve	+ve
Tannins	+ve	+ve
Saponins	+ve	+ve

Karamanoli *et al.* (2011) reported that tannins exert their action by combination of mechanism that includes iron chelation and enzyme inhibition. Though the exact mechanism behind the observed action of both plant powders is not yet known. Chaieb (2010) extensively reviewed insecticidal effects of saponins, linking their insecticidal activity with cholesterol which results in impaired ecdysteroid synthesis. Some of the reported observed effects of saponins are increased mortality, lowered food intake, weight reduction, retardation in development and decreased reproduction (Chaieb, 2010). Dolui *et al.* (2012), on the other hand, reported that tannin combined with protein to inhibit enzyme activity and reduce the availability of protein in haeolymph in insects. The ability of the plant powders to reduce the egg laying capability by the female beetles may be attributed to the presence of flavonoids in the plant. Righi-Assia *et al.* (2010) stated that flavonoids significantly reduced the egg laying and fertility in *C. Chinensis.* The insecticidal activity exhibited by the evaluated plants in this study correlates with the findings of Dolui *et al.* (2012) and Kannahi and Vinotha (2013) who previously reported the pesticidal potentials of the plants. In fact, complex mixtures of secondary compounds in plant extracts were

reported to contribute to a great deal for synergism, which enhances the joint action of active compounds against insect and reduces the rate of resistance development (Feng and Isman, 1995).

Adedire and Lajide (1999) and Sugita *et al.* (2013) reported 6-paradol, 6-gingerol and 6-shagaol (an alkyl phenol aromatic ketones) as the major insecticidal constituent of *A. melegueta* which is responsible for sharp and peppery taste of the seeds. While Olonisakin *et al.* (2007) reported the presence of β-pinene, β-phellandrene, γ-terpinene, eucalyptol and α-pinene as the predominant bioactive compounds responsible for the insecticidal activity of *X. aethiopica*, *A. melegueta* was reported to contain the following bioactive molecules: α-caryophyllene, β-caryophyllene, E-nerolidol, linalool, gingerdione, paradol, shagaol and humulene (Owokotomo *et al.*, 2014)

Findings from the present study confirm the plant bioactivity in suppressing oviposition and adult emergence of *C. maculatus* and thus are efficacious in protecting cowpea seeds from the insect infestation and damage at limited resource farmers' level and low volume seed storage. Since adult *C. maculatus* do not feed on stored cowpea seeds but only deposit their eggs, admixing the plant powders is recommended as ecofriendly and non-toxic methods in the management of *C. maculatus* for short duration storage. A more extensive study is necessary to determine the relative amounts of these materials quantitatively required for pest control strategy.

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A New Record of Two Species of Hydra in Iraq: An Ecological and Histological Study

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Abstract

Hydras are freshwater cnidarians. They are found on all the continents except Antarctica, and they are also found on Continental Island but are apparently absent from oceanic island. The two species of brown hydra, Hydra vulgaris (Pallas, 1766) and Hydra oligactis (Pallas, 1766) were recorded for the first time in Iraq from samples collected from two ponds in Erbil province Northern part of Iraq during May 2013. Some physical and chemical properties of water were studied among them; water temperature reached to 22.5 ºC, with neutral pH value most of other studied parameters indicated that water is clean, cool and not polluted.

Keywords: Cnidarian, Brown Hydra, fresh water, Iraq.

1. Introduction

Cnidarians are mostly marine animals; but they can be found in nearly all types of freshwater (i.e., streams, rivers, ponds and lakes), and occur mainly in mesotrophic to eutrophic habitats (Jankowski et al., 2008). Hydra, a freshwater polyp belonging to phylum Cnidaria and class Hydrozoa, is globally distributed except in the Antarctic region and Oceanic islands (Campbell, 1987).

Many previous studies were carried out on the hydra in different parts of the world. The first record of Hydra cauliculata was published by Hyman (1938) in which he reported the distributional notes of many other species especially Hydra littoralis in North America. Campbell (1999) recorded for the first time three species of hydra in Madagascar, which were Hydra viridissima, H. madagascarensis and Hydra sp. On the other hand, the first record of brown hydra, Hydra oligactis in Turkey was carried out by Şaşi and Balik (2002) in Topçam reservoir. Their study included a study of some ecological factors of reservoir water as temperature, dissolved oxygen, pH, transparency and conductivity. Reddy et al. (2011) conducted a study on the description and phylogenetic characterization of common hydra from India. While the presence of Hydra vulgaris for the first time in Los Padres Lagoon reservoir in Argentina was reported by Deserti and Zamponi (2011). Also, Deserti et al. (2011) published an investigation on Hydra genus in Argentina; the study included the main taxonomic characters of the four groups of hydra.

In Iraq, during the two last decades, many studies have been published on the different groups of invertebrates. However, there are no published information and no zoogeographic studies on cnidaria in Iraq. It is noteworthy that the present study records two species of Hydra for the first time in Iraqi inland water and gives their morphological description and key characteristics.

2. Materials and Methods

The study was made at two sites the one of small pond at Greater Zab River near Geaitly village and the other in a pond near Piran village, Erbil Governorate. At the study sites, the water depth varied from 15 cm near the banks to more than 2 m near the center, depending on local rainfall and water feeding from the river. The bottom was muddy and often filled with litter from overhanging trees (Fig. 1). Samples were taken on May (2013) in which *Ceratophyllum demersum* (Ceratophyllaceae) and attached submerged parts of *Typha angustifolia* L. macrophytes were taken out of from the bottom of the pond. The samples were stored in a package that contained water from the site and were transported to the laboratory. In the laboratory, the samples were placed in a glass aquarium that was 20 cm in diameter, with an aerator.

At the same time the samples were made, the main limnological parameters: water temperature, pH and electrical conductivity were taken in the field. In addition, the following analyses were done in the laboratory: dissolved oxygen, biochemical oxygen demand, chemical

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oxygen demand, orthophosphate, nitrate, nitrite and ammonium as described in (APHA 1998) (Table 1). Brown hydra was separated from the plant parts and was readily collected with a Pasteur pipette. Twice per week water was added from the pond. During this week, and under these conditions, measurements of the extended hydras were made using a micrometer under a microscope. The samples were identified according to Thorp and Covich (2001) and Schuchert (2010).

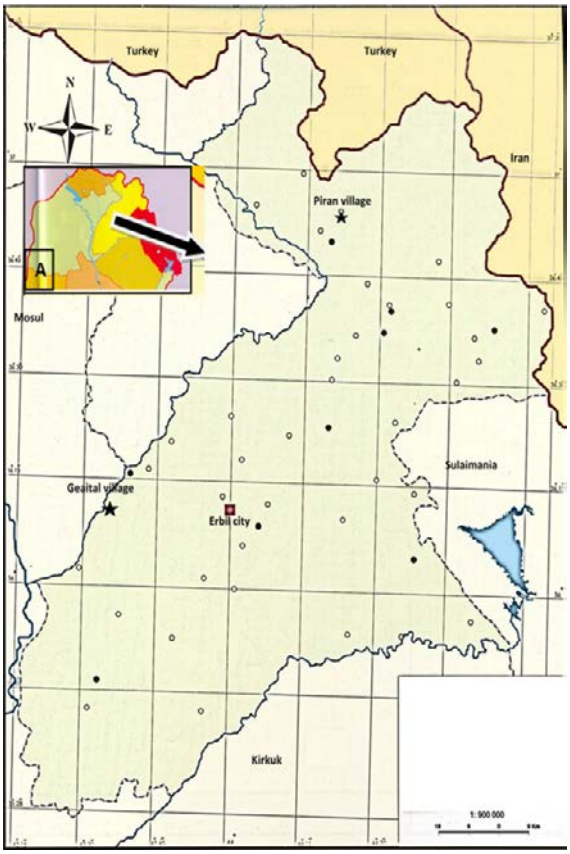

Figure 1. Map of Northern part of Iraq shows studied sites

3. Results and Discussion

3.1. Ecological factors

Brown hydra was observed in two locations, Geaitly village (about 30 km west of Erbil city) in a pond where our samples were collected and in Piran village (about 80 km north of Erbil city); both sites are characterized by clean water type. Water properties of the studied sites are found in (Table 1), water temperature reached to 22.5 ºC, with neutral pH value most of other studied parameters indicated that water is clean, cool and not polluted, and cleaner than others. Deserti and Zamponi (2011) and Campbell (1999) stated that the most consistent type of water in which hydra could be found is, for one reason or another, excluded from most human activities.

Table 1. Some physico- chemical water quality parameters of studied sites.

Ponds	Water Temp.	pH	EC ($\mu S.cm^{-1}$)	DO ppm	BOD_5ppm	COD ppm	$PO_4\mu g.l^{-1}$	$\mu g\ NO_2\text{-}N.l^{-1}$ NO_2	$mg\ NO_3\text{-}N.l^{-1}$ NO_3	$\mu g\ NH_4\text{-}N.l^{-1}$ NH_4
1	22.5	7.45	440	5.9	3.4	27	0.85	0.68	0.76	9.36
2	18.5	7.71	450	6.5	2.3	18	0.35	0.34	2.53	5.20

3.2. Description of Hydra Vulgaris Pallas, 1766

Polyps were brown in color, without a distinct stalk, the length of the column ranged from 1.97 to 9.88 mm and the width from 0.3 to 0.8 mm. The number of tentacles per polyp is 5-7, it was transparent and moniliform. Tentacles are shorter than body column reached up to 3/4 of the column. Hypostome was brown color and conical shape (Fig. 2).

3.2.1. Asexual Reproduction

Only one specimen displayed two buds at different development stages, they are located underneath the half of the column and they were brown in color. Tentacles emerged asynchronously (discontinuous) on buds, two tentacles emerged first, opposite each other, followed by two more perpendicular to the first pair, whereas the fifth on appeared randomly (Fig. 3). Buds has 5-7 tentacles.

3.2.2. Sexual Reproduction

They were not found.

3.3. Description of Hydra oligactis Pallas, 1766

Polyps were pale translucent brown in color, the length the column ranged from 15 to 25 mm with the base distinctly narrowed to form a stalk or foot, the number of tentacles per polyp is 6-9, it was transparent and moniliform. Tentacles are very long, which may extend to 5 cm or more when relaxed (Fig. 4). Hydra oligactis is dioecious, with males and females occurring as separate individuals.

3.3.1. Asexual Reproduction

Only one specimen displayed three buds at different development stages, they are located underneath the half of the column and they were translucent brown. Two lateral tentacles arising before the others on buds. Buds has 5-6 tentacles.

4. Nematocysts

The four characteristic nematocyst types were observed in both recorded species (Fig. 5).

Stenoteles are pear-shaped (pyriform), they were found in tentacles and in column. Its length 9.7 ± 1.35 µm and width 7.72 ± 1.12 µm.

The atrichous isorhiza were less abundant, and were present only in the tentacles. They are cylindrical in shape, 8 ± 0.7 µm length and 3.53 ± 0.3 µm width.

The holotrichous isorhiza were the least abundant nematocyst. They are paramecium-like and some cylindrical, 10 ± 0.45 µm length and 4.19 ± 0.29 µm width.

Desmonemes were the most abundant nematocysts. They were present in the tentacles only, and were pyriform in shape. Its length 6.26 ± 0.47 µm and width 4.43 ± 0.35 µm.

This group of hydra (brown hydra) was studied previously by different researcher in different parts of the world (Schulze, 1917; Campbell, 1989; Deserti and Zamponi, 2011; among others). However, in Iraq, there are no previous studies on this group; the present study is regarded the first one to record the two species of hydra in Iraq, particularly in Erbil province Northern part of Iraq. The description and measurements of the present specimens are nearly close to those reported by Thorp and Covich (2001) and Deserti *et al.* (2011).

Figure 2. Photographs of *Hydra vulgaris*, A. *H. vulgaris* with buds. B. Moniliform tentacles. C. Hypostome

Figure 3. The discontinuous growth of tentacles (10X)

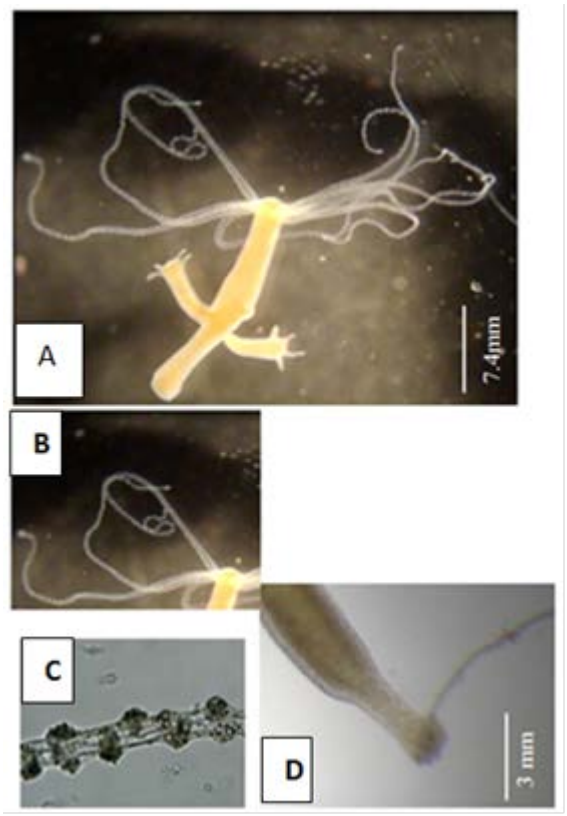

Figure 4. Photographs of *Hydra vulgaris*, A. *H. oligactis* with buds. B,C. Moniliform tentacles. D. Stalk (Foot).

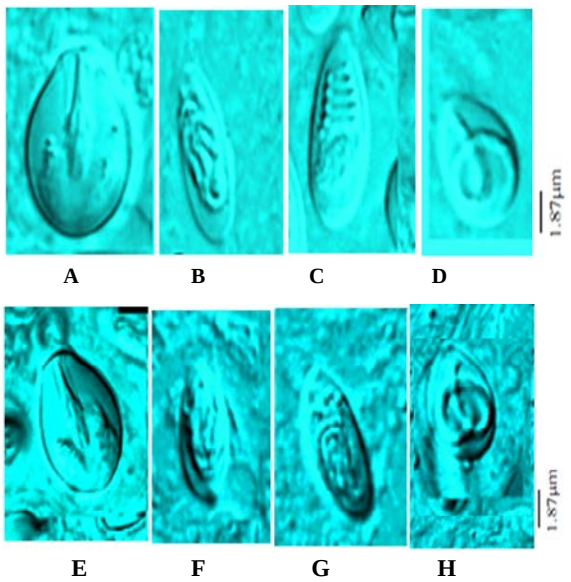

Figure 5. Type of Nematocysts: A-D *Hydra vulgaris*; **A**. Stenoteles, **B**. Atrichous isorhzas, **C**. Holotrichous isorhizas, **D**. Desmonemes. E-H *Hydra oligactis*; **E**. Stenoteles, **F**. Atrichous isorhzas, **G**. Holotrichous isorhizas, **H**. Desmonemes

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A Comparative Study of *in vitro* Antioxidant Activity and Phytochemical Constituents of Methanol Extract of *Aframomum melegueta* and *Costus afer* Leaves

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Abstract

Based on local claims on the efficacy of *Aframomum melegueta (alligator pepper)* and *Costus afer (ginger lily)* in the treatment of malaria, the present study compared the antioxidant activity and phytochemical constituents of extracts of *A. melegueta* and *C. afer* leaves. Methanol extracts of the plant leaves were obtained using standard procedures. The antioxidant property of the plants extracts were evaluated using DPPH (1,1-diphenyl-1-picryl-hydrazyl) radical, Total Phenol Content (TPC), Total Flavonoid Content (TFC), Proanthocyanidin Content (PC), Ferric acid Reducing Antioxidant Potential (FRAP) and ThioBarbituric Acid Reducing Substances (TBARS) assay. The phytochemical screening test revealed the presence of alkaloids, reducing sugars, flavonoids, tannins, saponins, steroids, cardiac glycosides and terpenoids in both extracts. The antioxidant study showed that the *A. melegueta* extract had higher DPPH radical scavenging ability (IC_{50} of 122.25ug/ml), FRAP (35.38µmol Fe(II)/g) and TBARS (% inhibition of 62.08%) than *C. afer* (IC_{50} of 156.48ug/ml, 12.25 µmol Fe(II)/g, and 42.5%, respectively). The *C. afer* extract, however, recorded higher levels of TFC, PC and a lower TPC content when compared with the *A. melegueta* counterpart. The results suggest that *C. afer* and *A. melegueta* extracts could serve as free radical scavengers, acting as primary antioxidants. The results support local claims of their therapeutic uses in the treatment of malaria in folklore medicine.

Keywords: *Aframomum melegueta*, *Costus afer*, phytochemical, antioxidant, Medicinal plant.

1. Introduction

Medicinal plants have been used in folk medicine for generations in most of the cultures throughout the world and are still one of the primary sources of treatment in many areas today. However, among the 250,000 - 500,000 species of plants on earth, only a relatively small percentage (1 - 10%) is used for food by humans and animals (Borris, 1996), while a higher percentage may serve medicinal purposes. The medicinal values of plants have been claimed to lie in their phytochemical components including alkaloids, tannins, flavonoids and other phenolic compounds, which produce a definite physiological action on the human body (Anyasor, 2011). Phytochemicals have been reported to protect the cell constituents against destructive oxidative damage, inhibition of hydrolytic and oxidative enzymes including lipid peroxidation, thus limiting the risk of various degenerative diseases associated with oxidative stress (Vinary *et al*., 2010).

Costus afer Ker-Gawl (Costaceae) is among 150 species of stout, perennial and rhizomatous herbs of the genus *Costus* (Edeoga and Okoli, 2000). It is found in the forest belt of Senegal, South Africa, Guinea, Niger, Sierra Leone and Nigeria (Burkill, 1985; Edeoga and Okoli, 2000). *C. afer*is commonly called bush cane, *irekeomode* (Yoruba) and *opete* (Igbo). It bears white and yellow flowers (Stentoft, 1988). The stem, seeds and rhizomes are harvested from the wild and contain several bioactive metabolites. *C. afer* is highly valued for its anti-diabetic, anti-inflammatory and anti-arthritic properties in South-East and South-West Nigeria (Soladoye and Oyesika, 2008). It is also widely used in Nigeria for the treatment of cough, malaria, venereal diseases, skin eruption and inflammation (Okoko, 2009).

*Aframomum melegueta*is a West African plant, with common (local) names such as "*alligator pepper*," "*guinea pepper*" and "grain of paradise." It is a member of the family Zingiberaceae and is locally called '*atare*' (Yoruba). It is widely spread across tropical rain forest regions of Africa including Nigeria, Liberia, Sierra Leone, Ghana, Cameroon, Cote D' Ivoire and Togo. The plant

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possesses both medicinal and nutritive values. The phytochemicals obtained from the seed has been used for years in the treatment of infectious diseases. The fruit pulp is chewed as a refreshing stimulant and the seeds and leaves are used for seasoning foods and in local medicine. It is also used as a remedy for a variety of ailments such as snakebite, diarrhea, smallpox, chickenpox, wounds, cough, anaemia, rheumatism, measles, malaria, toothache, cardiovascular diseases, diabetes and fertility control (Olowokudejo, 2008).

A number of studies have been conducted on the phytochemistry and pharmacology of the individual plant extract, but to the best of our knowledge there have been no comparative studies on the methanol extracts of both plants. Thus, the present study comparatively evaluates the phytochemical constituents and *in vitro* antioxidant activities of methanol extracts of *Costus afer* and *Aframomum melegueta* leaves..

2. Materials and Methods

2.1. Plant Materials

The leaves of Costus afer and Aframomum melegueta were collected from a farm in Edo State, Nigeria. The leaves were identified and authenticated by a Botanist in the Department of Plant Biology and Biotechnology, University of Benin.

2.2. Preparation of Crude Extract

The leaves of both plants were rinsed thoroughly with distilled water and allowed to dry under shade. The dried samples were then pulverized and stored in an air tight container. Two hundred grams (200 g) of each sample were extracted in 1000 mL of absolute methanol at room temperature for 72 hours. After that, each of the sample were filtered using Whatman No 50 filter paper and the filtrate evaporated to dryness using a rotary evaporator to give a percentage yield of 11.43 % (Costus afer extract) and 20.10 % (Aframomum melegueta extract). The resultant yields were stored in air-tight bottles and kept in the refrigerator maintained at 4oC until subsequent use.

2.3. Phytochemical Screening

Alkaloids, tannins, saponins, carbohydrates, anthraquinones, flavonoids and other phenolic compounds were screened qualitatively using the procedures previously described by Stahl (1973), Sofowora (1982) and Evans (2002).

2.4. Determination of Total Phenol Content (TPC)

The Total Phenolic Content (TPC) was determined according to the Folin and Ciocalteau's method using gallic acid as standard (Folin and Ciocalteau, 1927). Concentrations of 0.01- 1mg/ml of gallic acid were prepared in methanol. Concentrations of 1mg/ml extracts of Costus afer and Aframomum melegueta were also prepared in distilled water. About 0.5 mL of the sample was mixed with 2.5 mL of a ten-fold diluted Folin-Ciocalteau reagent and 2 mL of 7.5% sodium carbonate. The mixture was left undisturbed for 30 minutes at room temperature before the absorbance was read at 760 nm. All the determinations were performed in triplicates. The total phenolic content in the methanol extract was expressed in Gallic Acid Equivalents (GAE).

2.5. Determination of Total Flavonoids Content

The method of Miliauskas et al. (2004) was employed. To 2 mL of the sample was added 2 mL of 2 % $AlCl_3$ in methanol. The absorbance was read at 420 nm after incubation for 1 hour at room temperature. The concentration of 1 mg/ml of the extract in methanol was used, while quercetin concentrations ranging from 0.01 - 0.15 mg/ml were used to obtain the calibration curve. The total flavonoid content of the extract was expressed in Quercetin Equivalents (QE).

2.6. Estimation of Proanthocyanidin Content

The determination of proanthocyanidin was based on the procedure described by Sun et al. (1998). A volume of 0.5 mL of 1.0mg/ml of the extract preparation was mixed with 1 mL of 4 % vanillin-methanol solution and 0.75 mL concentrated hydrochloric acid. The mixture was left undisturbed for 15 minutes after which the absorbance was read at 500nm. The extract was evaluated at a final concentration of 1 mg/ml. The absorbance of ascorbic acid was read under the same conditions. Standard solution was prepared from 0.05g ascorbic acid. Total proanthocyanidin contents (mg/g) were expressed as ascorbic Acid Equivalents (AE).

2.7. Determination of DPPH Radical Scavenging Activity

The radical scavenging activity of the extract against 1, 1-diphenyl-1-picryl-hydrazyl radical (DPPH) was determined by a slightly modified method of Brand-Williams et al. (1995). The following concentrations of each extract were prepared in methanol at concentration ranging from 0.002 to 1mg/ml. Ascorbic acid was served as standard, and the same concentrations were prepared as the test solution. To 2 mL each of the prepared concentrations in a test tube was added 0.5 mL of 1mM DPPH solution in methanol. The experiments were carried out in triplicates. The test tubes were incubated for 15 minutes at room temperature, and the absorbance read at 517 nm. Ascorbic acid was used as a reference. The ability to scavenge DPPH radical was calculated by the following equation:

DPPH radical scavenging activity (%) = $[(A0-A1)/(A0)] \times 100$

where A0 was the absorbance of DPPH radical + methanol; A1 was the absorbance of DPPH radical + sample extract /standard.

2.8. Estimation of Thiobarbituric Acid Reactive Substances (TBARS)

A modified ThioBarbituric Acid Reactive Species (TBARS) assay was used to measure the lipid peroxide formed, using egg yolk homogenates as lipid rich media. Malondialdehyde (MDA), a secondary end product of the oxidation of poly unsaturated fatty acids, reacts with a pinkish red chromogen with an absorbance maximum at 532nm (Ruberto et al., 2000). Briefly 0.5 mL of Egg homogenate (10 %v/v) and 0.1 mL of extract were added to a test tube and made up to 1 mL with distilled water.Precisely 0.05 mL of FeSO4 (0.07M) was added to induce lipid peroxidation and incubated for 30minutes. Then 1.5 mL of 20% acetic acid(pH adjusted to 3.5 with

NaOH) and 1.5ml of 0.8%(w/v) TBA in 1.1% sodium dodecyl sulphate and 0.05 mL 20% TCA were added and the resulting mixture was vortexed, and, then, heated at 950C for 60 minutes. The generated color was measured at 523nm.

Inhibitions of lipid peroxidation (%) by concentrates were calculated with the formula:

(C-E) / C x 100 %

where C is the absorbance value of the fully oxidized control and E is (Abs523+Abs523-TBA).

2.9. Ferric Reducing Antioxidant Power (FRAP) Assay

The method employed was that of Benzie and Strain (1996) with a slight modification. Different concentrations (0.01, 0.025, 0.05, 0.075, 0.1, 0.15mg/ml) of the extracts and the standard were serially diluted with distilled water. Then, 1ml of FRAP reagent (200ml of 300mM sodium acetate buffer at pH 3.6, 20ml of 10.0mM TPTZ solution, 20ml of 20.0Mm FeCl3.6H2O solution and 24ml of distilled water) was added to each test tube. The resulting mixture was vigorously shaken and then incubated at 370C for 4mins and the increase in absorbance at 593nm was measured and compared with the standard ascorbic acid.

2.10. Statistical Analysis

All values were expressed as mean ± S.E.M. One way analysis of variance (ANOVA) was employed to assess the difference in mean between the groups. Turkey's multiple range post-hoc test was used to check the level of significance at p values less than 0.05

3. Results and Discussion

The therapeutic effects, derived from several medicinal plants, have been attributed to the presence of phenolic compounds such as flavonoids, phenolic acid, proanthocyandins and tannins (Pourmorad et al., 2006). Phenolics exhibit antioxidant activity by inactivating lipid free radicals or by preventing the decomposition of hydroperoxides into free radicals (Jimoh et al., 2008). These potential mechanisms make the diverse group of phenolic compounds an interesting target in the search for health beneficial phytochemicals (Akinpelu et al., 2010).In this study the phytochemical constituents and free radical scavenging activity of medicinal plants, were evaluated.

The result of phytochemical screening test of methanol extracts of *Aframomum melegueta* and *Costus afer* revealed the presence of medically active compounds including terpenoids, reducing sugars, flavonoids, saponins, alkaloids and cardiac glycosides in both leaves extracts. However, steroids and tannins were below the detectable levels in *Aframomum melegueta* and *Costus afer*, respectively (Table 1). Steroids have been reported with antibacterial properties and tannins have been found to have healing effects. Flavonoids are the most common group of polyphenolic compounds in the human diet and are found ubiquitously in plants where they exhibit antioxidant activities. Alkaloids are organic compounds that contain nitrogen, and are physiologically active with sedative and analgesic properties for relieving pains, anxiety and depression (Jisika et al., 1992). Therefore, the presence of these substances in the methanol extracts of *Costus afer* and *Aframomum melegueta* may be responsible for their therapeutic claims.

Figure 1 shows the total phenolic, flavonoid and proanthocyanidin contents of methanol extracts of *C. afer* and *A. melegueta*. Total phenolic content was expressed as mg gallic acid equivalent/g of extract by reference to a standard curve ($y = 0.001x + 0.033$; $R2 = 0.975$). The total flavonoid and proanthocyanidin content were expressed as mg quercetin equivalent/g of extract by reference to a standard curve ($y = 0.006x + 0.025$; $R2 = 0.996$) and mg ascorbic acid equivalent/g of extract by reference to standard curve ($y = 0.002x + 0.09$; $R2 = 0.915$), respectively. The results showed that *A. melegueta* extract had significantly higher ($p < 0.05$) concentration of total phenolic compounds than the *C. afer* extract, while the amounts of flavonoid and proanthocyanidin in *C. afer* were considerably high compared to *A. melegueta*. The different levels of antioxidant activities in these extracts may be due not only to differences in their phenolic contents, but also to their phenolic acid components (Horax et al., 2005). The presence of hydroxyl groups in the phenolic compounds may directly contribute to the antioxidant activity and is a key determinant of their radical scavenging and metal chelating activity (Amarowicz, 2004; Elmastas, 2007). Phenolic compounds could be a major determinant of antioxidant potentials of foods (Parr and Bolwell, 2000), and could therefore be a natural source of antioxidants (Aberoumand and Deokule, 2008). This finding is in agreement with Ukpabi *et al*. (2012) who state that extracts of *A. melegueta* and *C. afer* have considerably high amounts of polyphenols.

A number of methods are available for the determination of antioxidant capacity but the assay involving the stable 1, 1-Diphenyl-2-picrylhydrazyl radical (DPPH) has received the maximum attention due to its ease of use and its convenience (Sanchez-Moreno et al., 1998). The DPPH radical scavenging ability of the extract of *A. melegueta* showed maximum activity ($p < 0.05$) with IC_{50} of 122.25 µg/ml when compared with the *C. afer* counterpart (IC_{50} of 156.48 µg/ml). However, the standard reference compound, ascorbic acid gave a better DPPH scavenging ability than the two extracts with a lower IC_{50} of 118.55µg/ml. DPPH is a relatively stable free radical (Table 2). From the present result, it may be postulated that *A. melegueta* reduces the radical when it reacts with hydrogen donors in antioxidant principles. Also, when DPPH radicals react with suitable reducing agents, the electrons become paired off and the solution loses color stochiometrically depending on the number of electrons taken up (Ou, 2005). This is in agreement with Onoja et al. (2014) who state that *A. melegueta* seed has potent antioxidant activities when compared to standard ascorbic acid.

Our results in the present study showed that the ferric reducing antioxidant potential (FRAP) of methanol extract of *A. melegueta* (35.38µmole Fe (II)/g) was significantly higher ($p < 0.05$) than that of *C. afer* (12.25µmole Fe (II)/g). However, the FRAP values of both extracts were significantly ($p < 0.05$) lower than that recorded for standard ascorbic acid (140.5 µmole Fe(II)/g)

(Figure 3). Shiddhuraju et al. (2002) suggested that ferric reducing power of bioactive compounds was associated with antioxidant activity. It is a measure of the reductive ability of antioxidants and it is evaluated by the transformation of Fe^{2+} to Fe^{3+} in the presence of sample extracts (Huda-Faujan et al., 2009).

The methanol extracts of *C. afer* and *A. melegueta* leaves showed a varied degree of inhibition of lipid peroxidation induced by ferrous sulfate in egg yolk homogenates (Figure 4). The percentage inhibition of lipid peroxides by the methanol extract of *A. melegueta* (62.08 %) was significantly higher than that of *C. afer* (42.5 %). Lipid peroxidation contains a series of free radical mediated chain reaction processes and is also associated with several types of biological damages (Perry et al., 2000). These findings corroborate with Anyasor et al. (2014) who posited that the aqueous fractions of the leaves and stem bark of *C. afer* exhibited high inhibition of lipid peroxidation.

Our findings suggest that *C. afer* and *A. melegueta* extracts could serve as free radical scavengers, acting possibly as primary antioxidants which could be used in the treatment/management of disease caused as a result of oxidative damaged**.**

Table 1. Qualitative Phytochemical Screening of Methanol Extracts of *Costus afer* and *Aframomum melegueta* leaves

Phytoconstituents	*Aframomum melegueta*	*Costus afer*
Cardiac glycosides	+	+++
Steroids	-	+
Terpenoids	++	+++
Reducing sugars	+	+
Flavonoids	+	+
Tannins	+	-
Saponins	+	+
Alkaloids	++	+

Keys: - = Absent;+= positive;++ = moderately positive; +++ = highly positive

Table 2. IC_{50} values of *Costusafer and Aframomummelegueta* crude methanol extracts

Extract	IC_{50} value (µg/ml)
Ascorbic acid	118.55 ± 0.06^{a}
C.afer	156.48 ± 0.17^{b}
A.melegueta	122.25 ± 2.08^{c}

Data represent mean ± Standard Deviation of triplicate analysis. Different lowercase letters within column indicate significant difference at $p < 0.05$.

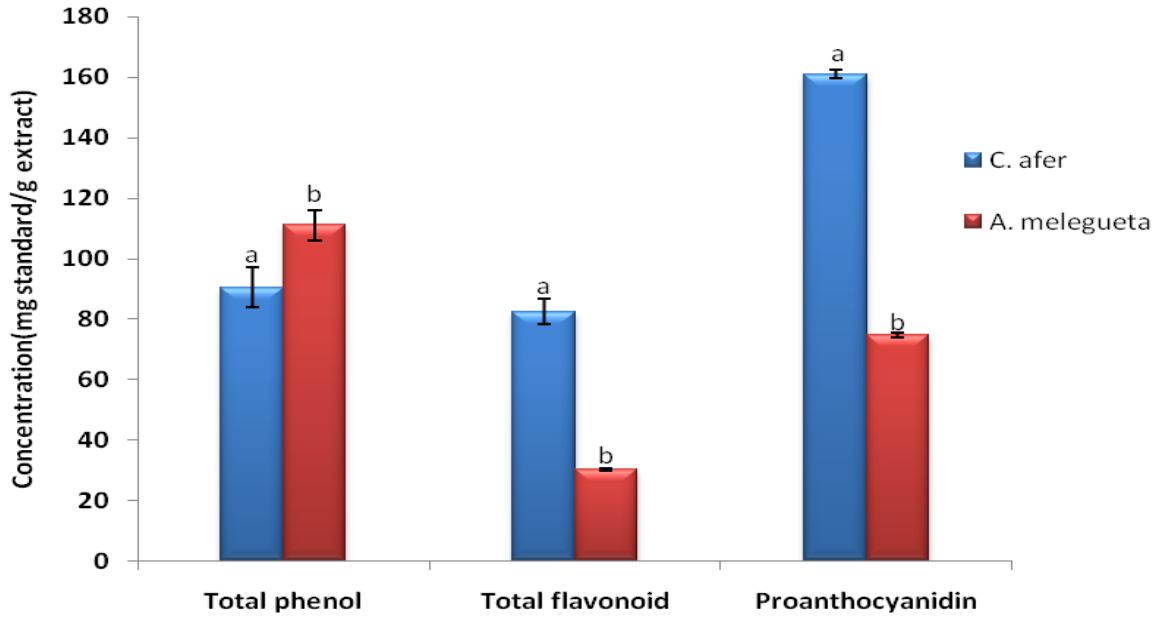

Figure 1. Polyphenolic contents of *Costus afer* and *Aframomum melegueta*

Values are expressed as mean ±SEM, *n* = 3/group. Different lowercase letters represent significant difference between means at $p > 0.05$.

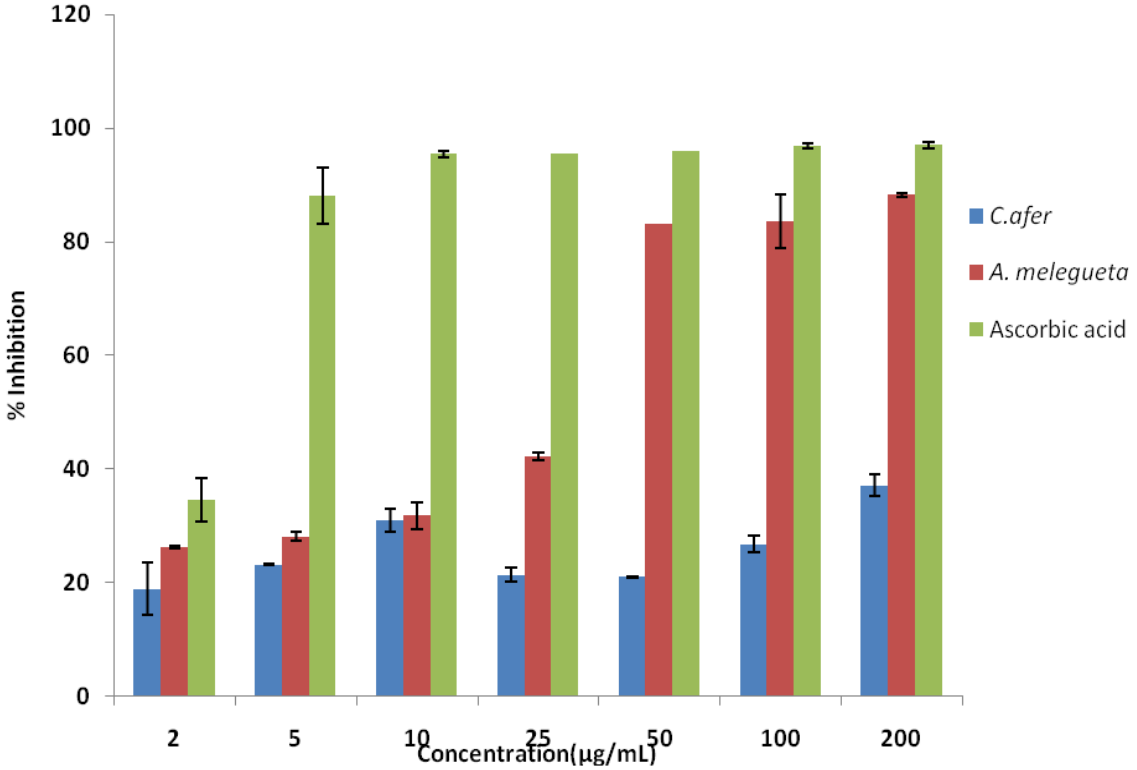

Figure 2. DPPH radical scavenging activity of *Costusafer and Aframomummeleguetacrude* methanol extract compared with standard (ascorbic acid) Data represent mean ± Standard Error of mean of triplicate analysis.

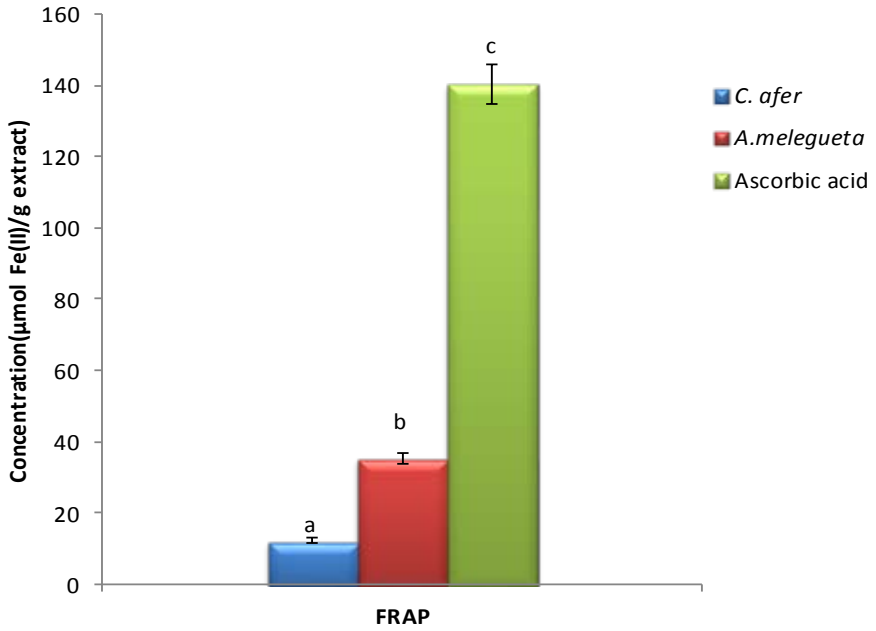

Figure 3. Ferric Reducing Antioxidant Potential (FRAP) of *Costusafer and Aframomummelegueta* crude methanol extracts

Values are expressed as mean ±SEM, *n* = 3/group. Different lowercase letters represent significant difference between means at P <0.05.

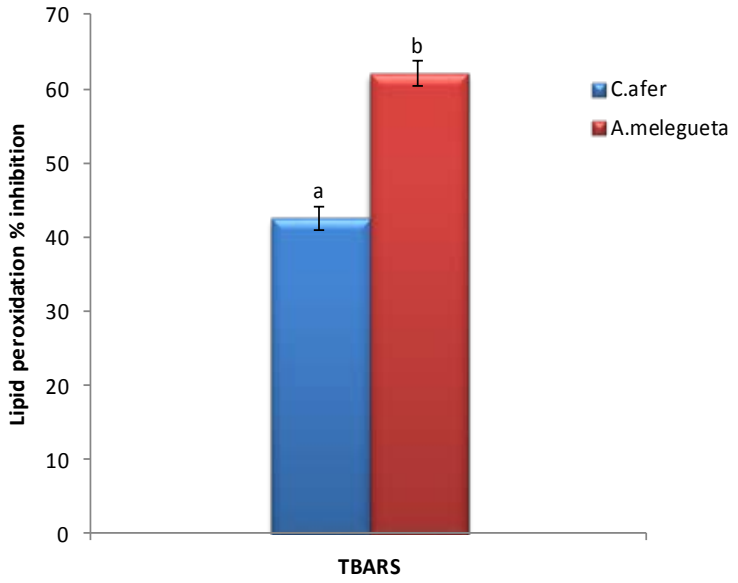

Figure 4. Thiobarbituric acid reactive substances (TBARS) of *Costus afer and Aframomum melegueta* crude methanol extracts
Values are expressed as mean± SEM, *n* = 3/group Different lowercase letters represent significant difference between means at P <0.05.

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Effect of Faba Bean (*Vicia faba* L.) Varieties on Yield Attributes at Sinana and Agarfa Districts of Bale Zone, Southeastern Ethiopia

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Abstract

Field experiments were conducted at Agarfa and Sinana Districts, south eastern part of Ethiopia with the objective of to evaluate high yielding faba bean varieties during the main cropping season of 2014. The following Eight improved faba bean varieties Mosisaa, Moti, Gebelcho, Hachalu, Shallo, Tumsa, Wolki and Degaga were used for evaluating their performance and the following data were recorded: effective tiller per plant, plant height, pods per plant, grain per pod, days to 90% physiological maturity, 100 grain weight, and grain yields were taken from the middle three rows and ten sample plants. The result revealed that there is a variation between the varieties for most yield and yield components. In both locations, maximum pods per plant were recorded from Degaga variety (20.39 and 22.6), whereas lowest number of pods were counted at Hachalu (12.46) and Tumsa (11.67) varieties at Agarfa site from Gebelcho variety (13.46) at Sinana experimental site. The maximum 100 grain weight was recorded from Gebelcho variety (94.33) at Sinana and Hachalu variety (80.33) at Agarfa experimental site. In both locations, the maximum harvesting index and grain yield were recorded at Shallo variety with 44.76%, 43.85% 4886.8 kg/ha and 4701.6 kg/ha at Agarfa and Sinana, respectively, while minimum grain yield was recorded from Hachalu and Moti varieties with 3436.2kg/ha and 3703.7kg/ha yield at Sinana and Agarfa district, respectively. Therefore, from the result we conclude that Shallo variety was performed good yield and recommended to the local farmers.

Keywords: *Faba bean varieties, Grain yield, yield attribute.*

1. Introduction

Faba bean (*Vicia faba* L.) is also referred to as broad bean, horse bean and field bean and it is the fourth most important pulse crop in the world (Sainte, 2011). The crop has a multipurpose use and is consumed as dry seeds, green vegetable, or as processed food. Its products are a rich source of high-quality protein in the human diet, while its dry seeds, green haulm and dry straw are used as animal feeds (Sainte, 2011). Faba bean seeds are used for human nutrition. The grain of faba bean contains a high protein content of 24-33% (Winch, 2006). Faba bean varieties that are used for human nutrition belong to the *V. faba* major botanically whereas the V. faba minor and *V. faba* equina are botanical types used for animal feeding (Monti et al., 1991).

Ethiopia is the world's second largest producer of faba bean next to China; its share is only 6.96 % of world production and 40.5 % within Africa (Chopra *et al*., 1989). In Ethiopia, the average yield of faba bean under small-holder farmers is not more than 1.6 t ha-1 (CSA, 2013), despite the availability of high yielding varieties (> 2 t/ha) (MoA. 2011). Demand is growing, fuelled by rapid population growth, and the gap between supply and demand continues to increase (ICARDA, 2008). In Ethiopia, the faba bean production is primarily a rain-fed system and it is also reducing the poverty by, 3% for adopter households moved up at least one 'wealth class', while all non-adopters remained in the lowest class (ICARDA, 2008).

Faba bean ranks first in pulse crop in the total area coverage and the total production of Ethiopia. It accounts about 36% of the country's pulse production (IFPRI, 2010). Currently, the total area, under cultivation with faba bean in the country, is estimated to be about 0.54 million hectare and the total production is 696 million kilogram (MoARD, 2009). Oromia region is the largest (320 million kilogram quintal) faba bean producer in the country followed by Amhara region 250 million kilogram (CSA, 2011). The two regions together share about 85% of the country's faba bean production. Due to its nitrogen fixing capacity, it is used in crop rotation with important cereal crops like wheat, teff, barley and other crops (IFPRI, 2010).

Farmers who adopted the new faba bean technologies, whether the full package or individual components, obtained significantly higher yields. Simply replacing traditional varieties with improved ones led to gains of 18% in Egypt, 8% in Sudan and 42% in Ethiopia (ICARDA, 2008). Levels of soil-borne diseases (like crown rot) and pests like root lesion nematode of wheat are reduced by growing faba bean as a break crop. Growers claim that the soil tilth is improved after faba bean growing (ICARDA, 2008).

The productivity of faba bean in Ethiopia is quite lower 15.2 qt/ha (CSA, 2011) than UK 30qt/ha (Winch, 2006). Most farmers in Ethiopia cultivate local faba bean varieties (Thijssen et al., 2008). Faba bean varieties, combining disease resistance with desirable traits like large seed sizes and high yield, are more preferred by the farmers (Boef and Ogliari, 2008). In Ethiopia, there are about 20 improved faba bean varieties which are adapted to different agro-ecology and have different disease reaction (IFPRI, 2010). Local varieties are low yielding and susceptible to both biotic and abiotic factors. Samuel et al. (2008) reported that most local faba bean landraces are highly susceptible to disease and low yielding. The reasons for the decline the productivity of faba bean is due to susceptibility to biotic (Sillero et al., 2010) and a biotic stresses (Link et al., 2010).

Bale District is one of the production areas of faba bean in Ethiopia for green and dry seed. Evaluation of high yielding variety is very important for farmers to sustain their production. The production of faba bean is affected by the lack of improved varieties and the local varieties are susceptible to certain biotic and abiotic factors. Bale area is a wheat belt of the country. The production of wheat is currently being challenged due to the monocropping practice which causes infestation and build-up of pests and diseases which in turn affect the productivity of wheat. This raises the issue of sustainability in livelihood as a result of which the tendency for food security can be jeopardized. Hence, growing of other alternative crop species like faba bean as a rotation crop is very important. Evaluation of improved faba bean varieties is among the major measures to be considered to resolve the existing problems of faba bean production and to boost the productivity of the crop. Therefore, the experiment was conducted to evaluate faba bean varieties for yield and yield components at Sinana and Agarfa District of Bale Zone.

2. Materials and Methods

The experiments were conducted at Sinana and Agarfa districts of Bale Zone, South-eastern part of Ethiopia during the main cropping season of 2014. The center represents highlands of Bale Zone with high rainfall and characterized by bimodal rainfall types. The main cropping season is locally known as *Ganna* extends, from half of July to September and the other cropping season was locally called *Bona* extends from March to July. In both locations, the dominant soil type is clay soil and slightly acidic with a Ph of 6.5.

Table 1. Geographical and Climatic condition of districts during cropping season.

District	Attitude	Temperature	Rainfall
Sinana	2361 – 2396	24.2°C to 9.4 °C	823mm
Agarfa	2404 – 2501	24.75°C to14.75°C	880mm

2.1. Treatments and Experimental Design

Eight improved faba bean varieties were collected from different research centers of Ethiopia and evaluated for their performance at Bale. The experiment was conducted by RCBD design with three replications and a plot size of 2.4m x 3.1m. To reduce the inter plot effect, the space between plots and blocks were adjusted at 0.5 and 1m, respectively. There were five rows per plot and 30 bean seeds were sown in a single row. Intra and inter spacing were 10 cm and 40 cm, respectively. The experiment was conducted with the following varieties: Wolki (EH96049-2), Degaga (R878-3), Moti (EH95078-6), Tumsa (EH99051-3), Hachalu (EH00102-4-1), Gebelcho (EH96009-1), Shallo (EH011-22-1) (MoARD, 2009) and Mosisaa (EH-99047-1) (SARC, 2013).

At the time of planting, all plots received a basal application of Diammonium Phosphate (DAP), (18% N, and 20% P) at the rate of 100 kg ha^{-1}. The experimental fields and experimental units were managed as per the recommended practices for faba bean.

2.2. Data Collection

The following data were collected from the middle three rows and from sampled ten plants in each plot.

- Productive tiller: Numbers of effective tillers were counted at poding stage.
- Plant height (cm): Length of the central axis of the stem, measured from the soil surface up to the tip of the stem.
- Number of pods per plant: was taken from 10 randomly selected plants from the middle rows of each plot at harvest.
- Number of grains per pod: Average numbers of grains per pod were counted at harvest.
- Hundred grain weight (g): sampled from the composited seeds of each plot and weighed after it had been adjusted to 10% moisture level by oven dry method.
- Days to 90% physiological maturity: it was recorded when 90% of the plants in the plot are mature enough.
- Dry Bio mass was harvested at crop maturity from the above ground part of net plot.
- Harvest index (HI) was calculated as the percent

$$HI = \frac{\text{Grain Yield}}{\text{Total biomass yield}} * 100$$

proportion of grain yield to total above ground DM yield (Fleischer *et al.* 1989).

- Grain yield (Kg/ha): The grain yield per hectare was determined by harvesting all plants from net plot area and converted on hectare basis.

2.3. Data Analysis

Collected data were subjected to analysis of variance with SAS computer software version 9.1.3 (SAS, 2003). Means were compared with Least Significance Difference

(LSD) at 5% probability level. The correlation analysis conducts as the procedure of SAS Proc corr. Procedure

3. Results and Discussion

The result from the experiment revealed that there is a variation between the varieties for various growth, yield and yield component parameters. However, there is no significance difference (P< 0.05) between the varieties for parameters like number of effective tillers, days to 50% flowering, days to 50% emergence and number of seeds per pod for both locations.

3.1. Number of Pods per Plant

Statistical analysis showed that faba beans varieties were significantly (P<0.05) affected pods per plant at both locations. The maximum pods per plant were recorded from Degaga (20.39 and 22.46, respectively, at Agarfa and Sinana) and it was followed by Shallo, Mosisaa, Moti and Tumsa in both locations Gebelcho at Agarfa experimental site and Hachalu at Sinana (Table 2 and 3). Whereas the smallest pods per plant were recorded at Tumsa and Hachalu varieties at Agarfa experimental site and Gebelcho variety at Sinana (11.67, 12.46 and 18.82) pods per plant, respectively. The result was in line with the work of Tafere *et al*. (2012) who reported that Degaga varieties had a higher number of pods per plant, while Gebelcho and Moti varieties had the smallest number of pods per plant. Hassan and Ishaq (1972) found that genotypes varied in their pod number per plant. Pilbeam *et al*. (1992) also reported that there was a variation between faba bean varieties for pod number per plant. Girma and Haila (2014) found that Degaga variety had the highest number of pods per plant while the result of Shallo variety was contradicting with this result which was lower in the number of pods per plant. The lowest number of pods per plant was recorded from Tumsa (11.67 and 18.82 at Agarfa and Sinana, respectively) and Hachalu varieties (12.46 and 16.46 at Agarfa and Sinana, respectively (Tables 2 and 3).

In this study number of pods per plant had a significant and positive correlation with seed per pod and days to 90% physiological maturity, while a negative correlation with plant height at both locations (Table 4).

Table 2. Effect of varieties on yield and yield component of faba bean at Agarfa site

Varieties	Tiller number	pod per plant	Seed per pod	Plant height	100g weight	Physiological Maturity	Dry matter Biomass (Kg/ha)	Harvesting Index %	grain yield Kg/ ha
Gebelcho	1.50a	15.67ab	3.00a	1.30a	80.33ab	126.6abc	11368.0abc	36.99bc	4362.2ab
Hachalu	1.45a	12.46b	2.92a	1.23a	83.10a	133.3a	9803.0c	40.95abc	4012.3b
Degag	1.50a	20.393a	2.96a	1.35a	56.13d	129.0ab	10190.4bc	39.25abc	3971.2b
Mosisaa	1.70a	16.29ab	3.04a	1.33a	64.12cd	119.67c	10167.9bc	42.9ab	4362.2ab
Moti	1.33a	13.29ab	3.29a	1.25a	79.60ab	119.67c	9935.0bc	37.38bc	3703.7b
Shallo	1.60a	16.75ab	3.04a	1.37a	60.48cd	124.3bc	10918.3bac	44.76a	4886.8a
Tumsa	1.40a	11.67b	3.04a	1.30a	79.80ab	130.67ab	11491.9ba	34.11c	3878.6b
Wolki	1.70a	17.21ab	3.04a	1.33a	69.33bc	133.3a	11865.3a	34.45c	4104.9b
LSD	0.37	5.67	0.45	0.16	11.01	7.04	18.71	10.24	760.25

Means with the same letter at the same column are not significantly different.

Table 3. Effect of varieties on yield and yield component of faba bean at Sinana site

Varieties	Tiller number	pod per plant	Grain Per pod	Plant height/m	100g weight	Physiological Maturity	Dry matter Biomass (Kg/ha)	Harvesting Index%	Grain yield (Kg/ ha)
Gebelcho	2.67a	13.46b	3a	1.28c	94.33a	128.3ab	10473.4ab	35.74ab	3703.7ab
Hachalu	2.79a	16.46ab	2.92a	1.33b	86.33ab	134.0a	8901.0b	38.61ab	3436.2b
Degag	2.92a	22.46a	2.96a	1.34b	59.13d	127.67ab	10029.0ab	37.58ab	3765.4ab
Mosisaa	2.96a	21.88a	3.04a	1.33bc	74.93bc	121.3b	10072.5ab	42.47a	4269.6ab
Moti	2.75a	16.54ab	3.29a	1.37ab	76.33bc	128.0ab	10059.7ab	36.4ab	3621.4b
Shallo	2.42a	20ab	3.04a	1.42a	65.77dc	126.67ab	10793.6ab	43.58a	4701.6a
Tumsa	2.75a	18.82ab	3.04a	1.35b	82.6b	128.0ab	11469.9a	30.81b	3497.9b
Wolki	2.33a	19.83ab	3.04a	1.38ab	66.07dc	134.67a	10670.0ab	38.48ab	4074.1ab
LSD	0.65	7.0	0.5	0.054	11.48	11.36	1959.6	15.82	1045.4

Means with the same letter at the same column are not significantly different.

3.2. Plant Height

Statistical analysis showed that plant height had significantly (P< 0.01) affected at Sinana where as non-significant at Agarfa experimental site. At Sinana experimental site, the maximum plant height was recorded from Shallo, Moti and Wolki varieties with a height of 1.42m, 1.37m and 1.38m, respectively. Whereas, the shortest plant heights were recorded at Gebelcho and Mosisaa varieties with 1.28m and 1.33m height, respectively (Table 3). Therefore, these varieties are considered as dwarf varieties. The result was in line with the work of Tafere et al. (2012) who reported that a Gebelcho verity was the shortest variety. Talal and Munqez (2013) reported that plant height was significant affected by faba bean accessions. Della (1988) found that plant height of faba bean genotypes varied significantly under rain fed conditions.

Plant height had a positive and significant correlation with pods per plant (r=0.54 and r=0.58) and a negative and significant correlation with seed per pod (r=-0.33 and -0.39), respectively at Sinana and Agarfa Districts.

3.3. Days to 90% Physiological Maturity

A significant difference (P<0.05) was observed among faba bean varieties for days to 90% physiological maturity at both locations. The maximum days to 90% physiological maturity was recorded from Wolki (134.7) and Hachalu (134.0) varieties at Sinana district. The minimum days to 90% physiological maturity was recorded from Mosisaa (121.3) and Shallo (126.7) varieties. On the other hand, the maximum days to 90% maturity at Agarfa district was observed from Wolki (133.3) and Hachalu (133.3) varieties, while the lowest was recorded from Mosisaa (119.7) and Moti (119.7) varieties. Generally, this result indicates that varieties like Hachalu and Wolki are late maturing varieties, while Mosisaa and Moti are early maturing varieties at both locations (Tables 2 and 3). Tafere et al. (2012) reported that Moti is early maturing variety while Gebelcho is late maturing. Talal and Munqez (2013) found that a significant variation was observed among accessions from days to fruit setting. The result disagrees with the work of Girma and Hail (2014) who found that faba bean varieties were non-significant on the physiological maturity in both irrigated and rain fed condition.

3.4. Hundred grain weight of Faba bean varieties

A hundred grain weight of faba bean was significantly (p<0.05) affected in both locations. The maximum 100 grain weight was recorded on Hachalu (83.1), Gebelcho (80.33) Tumsa (79.8) and Moti (79.6) varieties at Agarfa district. While, the lowest average 100 grain weight was recorded on Degaga and Shallo varieties with average 100 grain weight of 56.13 and 60.48, respectively (Table 2). On the other hand, the maximum 100 grain weight at Sinana was recorded from Gebelcho (94.33) and Hachalu (86.33) varieties, while the minimum 100 grain weight was from Degaga, Shallo and Wolki varieties with an average 100 grain weight of 59.13, 65.77 and 66.07, respectively (Table 3). This result revealed that Hachalu and Gabelcho varieties are large seeded, while Degaga and Shallo were small seeded varieties. Seed size is one of the important parameters for the choice of the varieties by the growers. Degaga variety has the largest number of seeds per pod, but it was small seeded; hence its yield was reduced (Tables 2 and 3). The result was in line with the work of Tamane et al. (2015) who reported that Moti, Tumsa and Gebelcho varieties were the higher 1000 grain weight while Degaga variety was the smallest grain weight. Girma and Haila (2014) reported that 1000 weight of Degaga variety was similar to Shallo variety and it was small.

3.5. Dry biomass

The statistical analyses showed that dry matter biomass had significant (p<0.05) different on faba bean varieties. The highest mean dry biomass weight (11469.9Kg/ha) recorded from Tumsa variety at Sinana district (Table 3). Similarly, the highest dry biomass weight was recorded from Wolki (11865.3Kg/ha) and Tumsa (11491.9Kg/ha) varieties at Agarfa district (Table 2). On the other hand, the lowest mean dry biomass was recorded from Hachalu variety with a dry biomass of 9935.0 and 8901.0 Kg/ha at Agarfa and Sinana District, respectively (Table 2 and 3). Abdalla *et al*. (2015)

reported that dry biomass was significantly varies with faba bean varieties. Comparatively, a higher dry biomass was noted from Agarfa District which may be attributed to the favorable conditions. Abdalla *et al*. (2015) and Toker (2004) reported the environments had significant effect on the dry biomass.

3.6. Harvest Index

In both locations, harvest index had significantly ($p<0.05$) affected on faba bean varieties. The maximum harvest index value was recorded from Shallo (43.58 and 44.76%) and Mosisaa (42.47 and 42.9%) varieties at Sinana and Agarfa District, respectively. Harvest index was significantly lower for Tumsa (30.81) variety as compared to Shallo variety at Sinana District, whereas there was no significant difference with the varieties (Tables 2 and 3). Similarly, the lowest harvest index was recorded from Tumsa (34.11%) and Wolki (34.45%) varieties at Agarfa District (Table 2). Gebremeskel *et al*. (2011) reported that the harvest index was significant for different faba bean varieties. Abdalla *et al*. (2015) also reported harvest index varies for different faba bean varieties. Contrasting results were reported by Agung and McDonald (1998) who stated that the harvest index differed little between genotypes. In this result, a higher grain yield producing varieties have a higher harvest index, while varieties which have a lower yield have a lower harvest index. A similar result was reported by Gebremeskel *et al*. (2011).

3.7. Grain Yield

The statistical analysis shows that a significant ($P<0.05$) difference was observed on grain yield of faba bean in both locations. In both locations, the maximum grain yield was harvested from Shallo variety which is (4886.8 kg/ha and 4701.6 kg/ha) at Agarfa and Sinana district, respectively (Tables 2 and 3). Similarly, the highest grain yield was obtained from Gebelcho (4362.2 kg/ha) and Mosisa (4362.2 kg/ha) varieties at Agarfa district, while the smallest grain yield was recorded at the varieties of Moti and Tumsa varieties with average grain yield of 3703.7 and 878.6 kg/ha (Table 2). Following Shallo variety higher yield was obtained from Mosisaa and Wolki varieties at Sinana district with average grain yield of 4269.6 and 4074.1 kg/ha, respectively. On the other hand, the minimum grain yield at Sinana district was recorded from Hachalu (3436.2) and Tumsa (3497.9 kg/ha) varieties (Table 3). The result was in line with Tafere et al. (2012) who reported that low grain yield was harvested on Moti varieties, but in the current finding Moti was a low yielding variety. ICARDA (2008) indicates that replacing of traditional varieties with improved ones led to gains of 18% in Egypt, 8% in Sudan and 42% in Ethiopia. Tamane et al. (2015) reported that Degaga variety was the higher grain yield as compared to Moti, Tumsa and Gebelcho varieties. ICARDA (2010) reported that Wolki variety can increase the grain yield up to 70-100% as compared to a local check. In the current finding, there is 24.2 % increment of yield for using Shallo variety (high yielder) as compared to Moti variety (low yielder) at Agarfa district. Similarly, there were 26.91% increments of yield for growing Shallo variety in place of Hachalu variety. The result contradicts the result of Girma and Haila (2014) who found that Degaga variety was a higher grain yield while Shallo variety was a lower grain yield. This variation may be due the environmental variations and the varieties adapting to different ecological conditions. ICARDA (2010) reported that Shallo variety was a high yielder. Fekadu (2013) reported that Roba-1 faba bean variety was reducing faba bean yield by 4.2%, whereas Awash-1 faba bean variety increasing faba bean yield by 24.3% as compared to the local check.

Combined analysis (Table 4) revealed that grain yield had positive and highly significant association with seed per pod ($r=0.84$ and 0.82) and dry biomass ($r=0.65$ and 0.61), respectively at Sinana and Agarfa Districts. This indicates that number of seed per pod had significant effect of grain yield of faba bean varieties.

Generally, there were correspondingly similar results for all faba bean varieties at both locations. However, except for the yield parameters there were larger values for most parameters were recorded from Sinana district. These indicate that Sinana site is more suitable for faba bean production.

Table 4. Correlation analysis among tiller number, pod per plant, seed per pod, Plant Height, 100grain weight physiological maturity, Dry Biomass, harvesting index and grain yield at Agarfa and Sinana District

	Parameter	Ti	Ph	Po	Se	Phi	Db
	Ti						
	Ph	0.28^{ns}					
	Po	-0.37*	0.54*				
Agarfa	Se	-0.18 ^{ns}	-0.33*	0.55*			
	Wt	-0.43*	-0.40 ^{ns}	- 0.63***	-0.11 ^{ns}		
	Phi	-0.19 ^{ns}	-0.11 ^{ns}	0.51*	-0.27 ^{ns}		
	Db	-0.51*	0.25 ^{ns}	0.12 ^{ns}	0.29 ^{ns}	-0.17 ^{ns}	
	Hi	0.28 ^{ns}	0.32 ^{ns}	0.27 ^{ns}	-0.29 ^{ns}	-0.51	-0.49**
	Gy	0.11 ^{ns}	-0.15 ^{ns}	0.15 ^{ns}	0.84**	-0.15 ^{ns}	0.65*
	Ti						
	Ph	0.25 ^{ns}					
Sinana	Po	-0.41*	0.58*				
	Se	-0.24 ^{ns}	-0.39*	0.59*			
	Wt	-0.42*	-0.21^{ns}	-0.59**	0.28 ^{ns}		
	Phi	0.026 ^{ns}	-0.41 ^{ns}	0.55*	0.1 ^{ns}		
	Db	-0.53*	0.28 ^{ns}	0.21 ^{ns}	-0.1 ^{ns}	-0.54*	
	Hi	-0.24 ^{ns}	0.37 ^{ns}	0.28 ^{ns}	-0.48 ^{ns}	-0.12 ^{ns}	-0.52*
	Gy	-0.019 ^{ns}	-0.30 ^{ns}	0.15 ^{ns}	0.82**	-0.14 ^{ns}	0.61*

Key: - Ti= tiller number, **Po**= pod per plant, **Se**=seed per pod, **Ph**= Plant Height, **Wt**= 100grain weight **Phi**=physiological maturity, **Db**= Dry Biomass, **Hi**= harvesting index and **Gy** = grain yield *P≤0.05, **P≤0.01, ***P≤0.001, ns= non-significant

4. Conclusion and Recommendations

The lack of improved varieties, which are well adapted to the farmers in the study area, was the main challenge for the farmers in the study area. Eight genetically modified varieties were evaluated for their yield and yield components faba bean at Bale Zone at Sinana and Agarfa districts. There were variations between the varieties for most of

The parameters and some varieties were performing well at both locations. The result indicates that there were slight variations for the varieties between the two locations. In both locations, the higher grain yield was harvested from Shallo variety which was followed by Gebelcho, Mosisaa and Wolki varieties where as Degaga variety was the lowest grain yield as compared to the other variety. Therefore, farmers located at Sinana and Agarfa Districts are recommended to use Shallo varieties to increase faba bean yield.

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The Phytochemical and Antimicrobial Properties of the Extracts Obtained from *Trametes elegans* Collected from Osengere in Ibadan, Nigeria

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Abstract

The phytochemical and antimicrobial properties of the extracts of the wild macrofungus, *Trametes elegans* (Spreng: Fr.) Fr. (fam.: Polyporaceae) obtained from a farm in Osengere, Egbeda local government area of Ibadan, Oyo State was investigated. The identity of the macrofungus was confirmed by amplifying and sequencing the Internal Transcribed Spacer (ITS 4 and ITS 5) of its nuclear ribosomal DNA (nrDNA). Extracts from the powdered fruiting bodies of the macrofungus were obtained by soaking in methanol and acetone. Antimicrobial effect of the macrofungus extracts was performed on clinical and referenced microbial cultures using standard microbiological techniques. In addition, phytochemical analysis of the *Trametes* species extracts was assessed qualitatively and quantitatively. The level of relatedness of *Trametes* species collected in Osengere and the already existing gene sequences of *Trametes* species in NCBI GenBank was 98%. Its closest relative is *T. elegans*. Phytochemical analysis revealed the presence of saponin, tannins, flavonoid, steroid, terpenoid, and cardiac glycosides in all the extracts with values ranging from 6.726 to 23.682 mg/g. The extracts of *T. elegans* displayed varying antimicrobial activities with zones of inhibition ranging from 1.50 to 25.50mm. The methanol extract of the macrofungus exhibited a better antibacterial activity against *Bacillus cereus* NCIB 6344 (20.5 mm), while the acetone extract of the macrofungus exhibited a higher antifungal activity against *Aspergillus fumigates* (25.5 mm). In light of this finding, *T. elegans*, collected from Ibadan, Oyo State, Nigeria, could be considered as potential sources of natural antimicrobial and could be of a great importance for the treatment of infectious diseases caused by the test organisms.

Keywords: Macrofungus, Trametes elegans, antimicrobial, phytochemicals, extracts.

1. Introduction

The need to explore natural sources for novel bioactive agents has increased in the last three decades. Fungi are among the most creative groups of eukaryotic organisms capable of producing many novel natural products that are directly used as drugs or serve as structural backbone for synthetic modifications (Mitchell *et al*., 2008; Stadler and Keller, 2008).

Fungi have been reported to be the second most diverse organisms, with a diversity (up to 3 to 5 million species) postulated to exceed that of terrestrial plants by an order of magnitude (Dai, 2010; Blackwell, 2011). Only a fraction of all fungal species has been described so far (about 100,000), and those explored for the production of important pharmacological metabolites are even less. Yet, some of the most successful drugs and agrochemical fungicides on the market have been developed from fungal secondary metabolites (De Silva *et al*., 2013). These include antibiotics (penicillins, cephalosporins and fusidic acid), antifungal agents (griseofulvin, strobilurins and echinocandins), cholesterol-lowering agents such as statin derivatives (mevinolin, lovastatin and simvastatin), and immunosuppressive drugs (cyclosporin) (Kozlovskii *et al*., 2013). Hence, the percentage of economically valuable fungal metabolites is still at large small.

Mushrooms are macrofungi with distinctive fruiting body, which can be hypogeous or epigeous, large enough to be seen with the naked eye and to be picked by hand (Chang and Miles, 1992). They are traditionally believed to be remedies for many diseases (De Silva *et al*., 2012) and are known to be prolific producers of bioactive metabolites (Wasser, 2011). Mushrooms have been used for centuries in Asia as popular medicines to prevent or treat different diseases (Xu *et al*., 2011; De Silva *et al*., 2012). The first record of the use of mushroom as

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hallucinogenic agent was credited to the Yoruba tribe of Nigeria in Africa (Griensven, 2009).

Macrofungi of the genus *Trametes* are polyporoid white rot fungi widely distributed in various biotopes and have been the subject of many physiological and biochemical studies (Iimura *et al*., 2002; Koroleva *et al*., 2002). They are capable of expressing a biological and a metabolic diversity as a result of their ability to utilize several substrates, decomposing dead organic matters, and, thus, to explore different habitat for colonization (Thatoi and Singdevsachan, 2014). Nigeria has unique climatic conditions and serves as home to diverse species of mushrooms. Yet, there is a dearth of information about these mushrooms and their medicinal potentials. According to Oyetayo (2011), factors such as mycophobia, trade secret by local herbalist, seasonal nature of mushrooms, and social stigma contribute to the underutilization of mushrooms in Nigeria.

Several authors have reported on the antimicrobial activities of different mushrooms, and have associated these activities to the presence of varieties of secondary metabolites such as peptides, tannins, terpenoids, phenols, and flavonoids (Alves *et al*., 2013; Lavanya and Subhashini, 2013; Avcı *et al*., 2014). The present study, therefore, aims to identify a *Trametes* species collected from Osengere, Egbeda Local government area of Ibadan, Oyo, Nigeria, using molecular tools, and to assess the phytochemical and antimicrobial properties of the macrofungus.

2. Materials and Methods

2.1. Collection of Macrofungus

Fresh fruit bodies of macrofungus suspected to be *Trametes* species were collected from rotten cocoa woods from farms in Osengere, Egbeda Local government area of Ibadan, Oyo State (Latitude:7.39814N, Longitude: 4.00051E) in August, 2013. The fruiting bodies were kept dry by wrapping in tissue paper and kept in a polythene paper containing silica gel. The polythene bags containing the samples were well labelled for easy identification and taken to the Department of Microbiology Laboratory of the Federal University of Technology, Akure for further morphological examination.

2.2. Molecular Identification of Macrofungus

2.2.1. Extraction of DNA of Macrofungus

Standard DNA isolation methods employing CTAB lysis buffer was used (Zolan and Pukkila, 1986). For DNA extraction, dried portions of the macrofungus fruiting bodies (2g) were ground with a mortar and pestle. The grounded materials were transferred into well labelled microtubes. Prewarmed (60^{o}C) extraction buffer (CTAB) was added and the tubes were incubated at 65^{o}C for 30 to 60 minutes. Equal volume of chloroform and alcohol (24:1) was added and mixed by inverting tubes for 15 minutes. The tubes were centrifuged for 10 minutes at 10,000g (13000rpm). The process was repeated but the time of mixing was 3 minutes and time of centrifugation was 5 minutes at the same speed as above. Upper aqueous layers were removed into clean tubes and 40µl 2M sodium acetate (NaAc) was added followed by 260µl of cold isopropanol. This was gently mixed by inverting tubes. The tubes were incubated at -20^{o}C overnight in a freezer (Haier HTF319 Freezer, 99405-0811, China). The second day, the mixture was centrifuged at 10,000g (13000rpm) for 10 minutes. The supernatant was discarded and pellets rinsed with 70% alcohol and mixed for sometimes. This procedure was repeated three times. After discarding the supernatant, the sample was dried in a dryer for 20 minutes at room temperature. Pellets were resuspended in 30µl Tris EDTA (TE) buffer (10 mM Tris/HCl + 1 mM EDTA pH 8). DNA concentration and quality was checked by observing the band on an ethidium-stained agarose gel (0.7%) using 0.2µl of each sample.

2.2.2. PCR Amplification of the ITS Region of macrofungus

The entire region of the rDNA of the macrofungus was amplified using the primers ITS4 and ITS5. The reaction mix was made up to a total volume of 25µl, composed of 23µl of *Taq*polymerase "Ready to Go" (Pharmacia, Sweden) with 0.2 µl of each primer (100 pM) and 2µl of DNA solution. The amplification reactions were performed in a DNA Thermal Cycler (GenAmp OPCR System 2400; Perkin–Elmer, USA) and programed as follows: 1st cycle of 5 min at 95°C (initial denaturation) followed by 30 cycles of 45 sec at 95°C (denaturation), 30 sec at 50°C (annealing), 1 min at 72°C (extension), and 1 cycle of 10 min at 72°C (final extension). The amplification products were purified using a PCR Purification Kit (USA) and electrophoresed on ethidium stained agarose gel (0.7%) to check the purity. DNA sequence was performed using the same primer pair used in the PCR reactions (ITS4 and ITS5) in an Applied Biosystem DNA Analyser (USA).

2.2.3. Sequencing of DNA and Alignment of Sequence

Alignments were performed with the Clustal W package (Thompson *et al*., 1997). The aligned sequences were corrected manually, focusing on gap positions. DNA sequence data were analysed to provide pairwise percentage sequence divergence. The data obtained from the sequence alignment were used to plot a tree diagram (MEGA 4 Software). Extraction, amplification and sequencing of rDNA were carried out at the Key Laboratory of Mycology and Lichenology, Institute of Microbiology, Beijing.

2.3. Collection of Test Organisms

Typed cultures (*Escherichia coli* ATCC 23718, *Escherichia coli* ATCC 35218, *Pseudomonas aeruginosa* ATCC 29853, *Bacillus cereus* NCIB 6344, *Staphylococcus aureus* NCIB 950, *Salmonella typhi* ATCC 33458) were collected from the Medical Microbiology Laboratory of the University College Hospital (UCH) Ibadan, Oyo state. Clinical isolates of human origin (*Bacillus cereus*, *Pseudomonas aeruginosa*, *Salmonella typhi*, *Staphylococcus aureus*, *Escherichia coli*, *Enterococcus faecalis*, *Candida albicans* and Methicillin Resistant *Staphylococcus aureus* (MRSA) were collected from Obafemi Awolowo University

Teaching Hospital (OAUTH), Ile-Ife, Osun state. *Aspergillus fumigatus*, *Aspergillus niger* were obtained from the culture collection of the Department of Microbiology, FUTA. The isolates were tested for viability by resuscitating them in buffered peptone water after which they were subcultured on nutrient agar medium for bacteria and potato dextrose agar for fungi and incubated at 37°C for 24 hours and 27°C for 48-72 hours, respectively. The organisms were then stored at 4°C until needed.

2.4. Preparation of Mushrooms Extracts

The collected fresh mushrooms were brush-cleaned of attached soil and humus and then air-dried in an oven at 40°C for 48 hours. They were then cut into bits, pulverised by an electrical mill, and stored in an air-tight container for further use. The powdered mushroom sample (100g) was extracted by maceration in 2000ml of 95% acetone and methanol separately in an Erlenmeyer flask. The flasks were covered with aluminium foil and allowed to stand for 3 days for extraction with occasional stirring. The extracts were then filtered through Whatman filter paper (0.45µm) using vacuum pump. The filtrates were evaporated to dryness at 50°C in a rotary evaporator (RE-52A; Union Laboratory, England) with 90 rpm under reduced pressure. The obtained concentrated extracts were stored in dark at 4°C until further analysis. The percentage yield of extracts was calculated based on dry weight as follows:

$$\text{Yield } (\%) = \frac{\text{W1 x 100}}{\text{W2}}$$

where,

W1= weight of extract after solvent evaporation

W2 = Weight of the grounded mushroom powder

2.5. Phytochemical screening of Trametes species extracts

A qualitative and quantitative phytochemical analysis of the crude mushroom extracts was performed through standard protocols described by Odebiyi and Sofowora (1978), Trease and Evans (2005), and Harborne (2005).

2.5.1. Qualitative phytochemical analysis of Trametes species extracts

2.5.1.1. Test for alkaloids

The extracts (0.5g each) were stirred with 5ml of 1% aqueous hydrochloric acid (HCl) for two minutes on a steam water bath. The mixtures were filtered and few drops of Dragendorff's reagent were added. The samples were then observed for color changes or turbidity to draw inference.

2.5.1.2. Test for saponins

The persistent frothing test for saponin described by Odebiyi and Sofowora (1978) was used. Distilled water (30ml) was added to 1g of each of the mushroom extracts. The mixture was vigorously shaken and heated on a steam water bath. The samples were observed for the formation of froth to draw inference.

2.5.1.3. Test for phlobatannins

The mushroom extracts (0.2g) were dissolved in 10ml of distilled water each and filtered. The filtrates were boiled with 2% HCl solution and observed for deposition of red precipitate which indicates the presence of phlobatannin.

2.5.1.4. Test for tannins

The method of Trease and Evans (2005) was adopted. Each sample (0.5g) was dissolved in 5ml of distilled water, then, boiled gently and cooled. One ml of each solution was dispensed in a test tube and 3 drops of 0.1% ferric chloride solution were added and observed for brownish green or blue black coloration which indicates the presence of tannins.

2.5.1.5. Test for terpenoids

The Salkowski test was used. Five ml of each extract weres mixed in 2 ml of chloroform, and 3 ml concentrated sulphuric acid (H_2SO_4) were carefully added to form a layer. Each solution was then observed for reddish brown coloration which confirms the presence of terpenoids.

2.5.1.6. Test for steroids

Acetic anhydride (2ml) was added to 0.5g of each extract and filtered. Sulphuric acid (2ml) was added to the filtrate and observed for color change from violet to blue or green, which indicates the presence of steroid.

2.5.1.7. Test for flavonoids

Diluted ammonia solution (5ml) was added to portions of aqueous filtrate of each mushroom extracts. This was then followed by the addition of a concentrated sulphuric acid. The solutions were observed for yellow coloration that disappears on standing to confirm the presence of flavonoids.

2.5.1.8. Test for anthraquinones

Borntrager's test was used for the detection of anthraquinone. The extract (0.5g) was shaken with 10ml of benzene, filtered and 5ml of 10% ammonia solution added to the filtrate. The mixture was shaken and observed for the presences of pink red or violet color in the ammonia layer which indicates the presence of free anthraquinones.

2.5.1.9. Test for cardiac glycosides

The following was carried out to test the cardiac glycosides of each extract.

2.5.1.9.1. Legal's test

Each extract was dissolved in pyridine and a few drops of 2% sodium nitroprusside with a few drops of 20% NaOH were added. They were observed for a deep red coloration which fades to a brownish yellow indicating the presence of cardenolides.

2.5.1.9.2. Salkowski's test

Each extract was mixed with 20ml of chloroform and filtered. This was followed by the addition of 3ml of conc. H_2SO_4 to the filtrate to form a layer. A reddish brown color at the interface was observed which indicates the presence of steroidal ring.

2.5.1.9.3. Keller- Killiani's test

Each of the extracts (0.5g) was dissolved in 2ml of glacial acetic acid containing one drop of ferric chloride solution. This was then underlayed with 1ml of conc. H_2SO_4. It was observed for a brown coloration at the interface indicating the presence of a deoxy sugar which is a characteristic of cardenolides. It was also observed for violet ring which may appear below the brown ring while in the acetic acid layer. The presence of a green ring formed just above the brown ring which can gradually spread throughout this layer, also indicates the presence of cardiac glycosides.

2.5.2. Quantitative phytochemical screening of Trametes species extracts

2.5.2.1. Determination of Tannins

Tannins determination was done according to the method of Association of Official Analytical Chemists [AOAC] (1990), with some modifications. The sample (0.20 g) was mixed with 20 ml of 50% methanol. This was shaken thoroughly and placed in a water bath at 80°C for 1 hour to ensure uniform mixing. The mixture was filtered into a 100ml volumetric flask, followed by the addition of 20ml of distilled water, 2.5 ml of Folin-Denis reagent and 10 ml of 17% aq. Na_2CO_3 (Sodium carbonate) and was thoroughly mixed. The mixture was made up to 100 ml with distilled water, mixed and allowed to stand for 20 min. The absorbance of the tannic acid standard solutions as well as sample was measured after color development at 760 nm using the spectrophotometer (Gulfex Medical and Scientific England, Spectrum Lab 23A, model number 23A08215). Results were expressed as mg/g of tannic acid equivalent using the calibration curve: $Y = 0.0593x - 0.0485$, $R = 0.9826$, where x is the absorbance and Y is the tannic acid equivalent.

2.5.2.2. Determination of saponins

Quantitative determination of saponins was done using the method of Obadoni and Ochuko (2001). The powdered sample (20 g) was added to 100 ml of 20% aqueous ethanol and kept in a shaker for 30 min. The samples were heated over a water bath for 4 hours at 55°C. The mixture was then filtered and the residue re-extracted with another 200 ml of 20% aqueous ethanol. The combined extracts were reduced to approximately 40 ml over the water bath at 90°C. The concentrate was transferred into a 250 ml separatory funnel and extracted twice with 20 ml diethyl ether. The ether layer was discarded while the aqueous layer was retained and to which 60 ml n-butanol was added. The n-butanol extracts were washed twice with 10 ml of 5% aqueous sodium chloride. The remaining solution was heated on a water bath. After evaporation, the samples were dried in the oven at 40°C to a constant weight.

2.5.2.3. Determination of alkaloids

Alkaloids were quantitatively determined according to the method of Harborne (2005). Two hundred milliliters of 10% acetic acid in ethanol were added to 5g powdered extract, covered and allowed to stand for 4 hours. The filtrate was then concentrated on a water bath to one-fourth of its original volume. Concentrated ammonium hydroxide was added dropwise to the extract until the precipitation was completed and the whole solution was allowed to settle. The collected precipitates were washed with dilute ammonium hydroxide and then filtered. The residue was dried and weighed. The alkaloid content was determined using the formula:

$$\text{Alkaloid (\%)} = \frac{\text{final weight of sample}}{\text{initial weight of extracts}} \text{ x 100}$$

2.5.2.4. Determination of steroid

The steroid content of the plant sample was determined using the method described by Trease and Evans (2005). A portion of 2ml was taken from a solution of 2.5g of powdered plant material prepared in 50mL of distilled water after vigorous shaking for 1 hour. The extract solution was washed with 3mL of 0.1M NaOH (pH 9) and later mixed with 2mL of chloroform and 3mL of ice cold acetic anhydride followed by the cautious addition of two drops of concentrated-H_2SO_4. The absorbance of both sample and blank were measured using a spectrophotometer (Gulfex Medical and Scientific England, Spectrum Lab 23A, model number 23A08215) at 420 nm.

2.5.2.5. Determination of cardiac glycosides

Cardiac glycoside content in the samples was evaluated using Buljet's reagent as described by El-Olemy *et al.* (1994). The samples were then purified using lead acetate and disodium hydrogen phosphate (Na_2HPO_4) solution before the addition of freshly prepared Buljet's reagent (containing 95ml aqueous picric acid + 5ml 10% aqueous NaOH). The difference between the intensity of colors of the experimental and blank (distilled water and Buljet's reagent) samples gives the absorbance and is proportional to the concentration of the glycosides.

2.6. Determination of antimicrobial activity of Trametes species extracts

Antimicrobial activity of extracts was determined by the agar well diffusion method described by Schinor *et al.* (2007). Stock cultures were maintained at 4°C on nutrient agar slope. Active cultures for experiments were prepared by transferring a loopful of cells from the stock cultures to test tubes of Nutrient Broth (NB) for bacteria and Sabouraud Dextrose Broth (SDB) for fungi and were incubated without agitation for 24 hours at 37°C and 25°C, respectively. To 5ml of NB and SDB, 0.2 ml of culture was inoculated and incubated till it reached the turbidity equal to that of the standard 0.5 McFarland solution at 600nm which is equivalent to 10^6– 10^8 CFU/ml.

Suspensions of fungal spores were prepared from fresh mature (5 days) cultures that grew at 26 ± 1ºC on a Sabouraud dextrose agar. Spores were rinsed with sterile distilled water. The suspensions were then adjusted to 10^6 spores per ml by microscopic enumeration with a cell counting hematocytometer. Molten Mueller Hinton agar (20ml) cooled to 45°C was poured into sterilized petri dishes and left to solidify. An aliquot of culture (100µl) was evenly spread on the surface of the solidified Mueller Hinton agar plates. Wells of 8 mm were bored in the agar

with sterile cork borers (6 and 7mm). The crude extracts (100µl) were dissolved in 30% dimethylsulfoxide (DMSO) to a concentration of 50 mg/ml and filtered through 0.22µm membrane filter and then introduced into each wells with the aid of a micropipette.

Clotrimazole was used as a positive control for fungi and reference antibiotic disc (gentamicin (10µg), nalidixic acid (30µg), nitrofurantoin (200µg), cotrimoxazole (25µg), amoxicillin (25µg), tetracycline (25µg), augmentin® (30µg), and ofloxacin (5µg) for bacteria. DMSO (30%) was used as negative control. The plates were allowed to stand for one hour at room temperature (26 ± 2ºC) to allow a proper diffusion of the extracts. The plates were then incubated at 37 ºC for 24 hour for bacteria while the fungi were incubated at 26 ± 1ºC for 48 to 72 hour. Inhibition zones were measured with a ruler in triplicates (three plates per indicator organism).

2.6.1. Determination of minimum inhibitory concentration

The agar diffusion method, described in section 2.6, was used to screen the antimicrobial effect of the different concentrations of extracts (6.25 to 50mg/ml). The MIC was determined by establishing a visible growth of microorganisms. The boundary dilution without any visible growth was defined as the MIC for the tested microorganism at the given concentration. DMSO solution (30%) was used as a negative control. The tests were performed in triplicates.

2.7. Statistical analysis

All experiments were carried out in triplicates. Data obtained were analyzed by One way analysis of variance (ANOVA) and means were compared by New Duncan's Multiple Range Test (SPSS version 16). Differences were considered significant at $p<0.05$.

3. Results

The Internal Transcribed Spacer (ITS) region of the macrofungus discriminated between the sequence of *Trametes* species collected from Osengere, Oyo State and the existing genes of *Trametes* species obtained from National Center for Biotechnology Information (NCBI) GenBank. *Trametes elegans* (Spreng: Fr.) Fr. (fam.: Polyporaceae) (Plate 1) with the ascession number (JN048766.1) was seen to be the closest relative to the *Trametes* species collected from Osengere with a percentage relatedness of 98 (Table 1).

Methanol produced a higher yield than acetone when used for extraction (Table 2). Results from the phytochemical analysis revealed the presence of saponins, tannins, steroids, terpenoids, flavonoids and cardiac glycosides in all the *Trametes* species extracts, while anthraquinones, alkaloids and phlobatannins were absent (Table 3).

Plate 1. A- Fruiting body of *Trametes* species collected from rotten woods forest in Osengere, Egbeda Local government area of Ibadan, Oyo State

Table 1.Genomic identification based on nrDNA Internal Transcribed Spacer (ITS) sequence of wild macrofungus collected from Ibadan, Nigeria.

Sample	Source	Tentative phenotypic identity	Closest relative from NCBI GeneBank	NCBIAccession numberOf closest relative	% identity
1	Ibadan, Oyo State	*Trametes* sp.	*Trametes elegans*	JN0 48766.1	98

Keys: NCBI-: National Center for Biotechnology Information**; nrDNA-:** Nuclear Ribosomal Deoxyribonucliec Acid

Table 2. Yield of *Trametes* species extracts

Mushroom	Solvent	Total yield (mg/g)
Trametes elegans	Methanol	3.1
	Acetone	0.9

Table 3. Qualitative phytochemical screening of *Trametes elegans* extracts

Phytochemical	Extracts	
	TEA	TEM
Saponin	+	+
Tannin	+	+
Steriod	+	+
Alkaloid	-	-
Terpenoid	+	+
Flavonoids	+	+
Anthraquinone	-	-
Phlobatannin	-	-
Cardic Glycoside		
1. Legal Test	+	+
2. Keller Killiani Test	+	+
3. Salkwoski Test	+	+

Keys: TEA: Acetone extract of *Trametes elegans***; TEM:** Methanol extract of *Trametes elegans***; +:** Positive**; - :** Negative.

The values of the various phytochemicals present in the extracts ranged from 6.726 to 23.682 mg/g (Table 4). Table 4 also shows that the methanol extract of the macrofungus has the highest amount of cardiac glycosides (23.682mg/g), flavonoids (7.290mg/g), saponins (18.000mg/g) and tannins (11.943mg/g), while the acetone extract of the macrofungus has the highest amount of steroids (14.546mg/g) and terpenoids (22.285mg/g).

Table 4. Quantitative phytochemical components of *Trametes elegans* extracts

Phytochemical	Amount (mg/g)	
	TEA	TEM
Tannin	9.665 ± 0.004^{b}	11.943 ± 0.0199^{c}
Saponin	16.163 ± 0.107^{d}	18.000 ± 0.105^{e}
Flavonoid	6.726 ± 0.004^{a}	7.290 ± 0.023^{a}
Steriod	14.546 ± 0.008^{c}	14.290 ± 0.031^{d}
Terpenoid	22.285 ± 0.008^{f}	10.545 ± 0.016^{b}
Glycoside	20.583 ± 0.056^{e}	23.682 ± 0.018^{f}

Keys: TEA: Acetone extract of *Trametes elegans***; TEM:** Methanol extract of *Trametes elegans***.**

Each value is expressed as mean ± standard error (n = 3). Values with different superscript within a column are significantly different at ($p < 0.05$).

Table 5 shows the antimicrobial activities of the extracts of *T. elegans* (50mg/ml) and commercial drugs against the test organisms. For the clinical bacterial isolates, the extracts could only inhibit *E. coli*, *P. aeruginosa* and *E. faecalis*. Methanol extract of *T. elegans* was able to inhibit all the referenced bacteria except for *P. aeruginosa* ATCC 29853, while the acetone extract was found to inhibit *E. coli* ATCC 23718 and *S. typhi* ATCC 33458. The methanol extract had no inhibitory effect on the test fungi, while the acetone extract inhibited all the test fungi except for *A. flavus*. Of the commercial antibiotics used, ofloxacin (5µg) gave the highest antibacterial activity, which was closely followed by nalidixic acid (30µg). The commercial antifungal drug, clotrimazole (1mg/ml) also inhibited all the test fungi. The activities of the commercial drugs when compared to those of the extracts were slightly higher and significantly different ($P< 0.05$). The Minimum Inhibitory Concentration (MIC) of the extracts ranged from 12.5 to 50mg/ml, while with the referenced bacteria exhibiting lesser MIC values (Table 6).

Table 5: Antimicrobial activities of extracts of *Trametes elegans* and commercial drugs against test organisms

Test organisms	Zones of inhibition (mm)						
	TEM (50mg/ml)	TEA (50mg/ml)	Gentamicin (10µg)	Nalidixic acid (30µg)	Nitrofurantoin (200µg)	Ofloxacin (5µg)	Clotrimazole (1mg/ml)
*Escherichia coli**	6.33 ± 0.17 ^{b}	6.00± 0.00 ^{b}	10.00 ± 0.58 ^{c}	12.33 ± 1.20 ^{d}	0.00 ± 0.00 ^{a}	21.33 ± 0.88 ^{e}	ND
*Pseudomonas aeruginosa**	9.50 ± 0 .29 ^{b}	4.17 ± 0.17 ^{a}	12.00 ± 0.29 ^{c}	15.83 ± 0.17 ^{d}	16.17 ± 0.44 ^{d}	20.00 ± 0.00 ^{e}	ND
*Bacillus cereus**	0.00 ± 0.00 ^{a}	0.00 ± 0.00 ^{a}	12.33 ± 1.20 ^{b}	18.67 ± 1.76 ^{d}	17.00 ± 0.58 ^{cd}	14.33 ± 0.88 ^{bc}	ND
*Staphylococcus aureus**	0.00 ± 0.00 ^{a}	0.00 ± 0.00 ^{a}	12.00 ± 2.00 ^{b}	16.00 ± 2.00 ^{c}	11.00 ± 1.00 ^{b}	20.67 ± 2.08 ^{d}	ND
MRSA*	0.00 ± 0.00 ^{a}	0.00 ± 0.00 ^{a}	11.33 ± 0.88 ^{b}	19.00 ± 0.58 ^{c}	11.33 ± 0.88 ^{b}	25.00 ± 1.16 ^{d}	ND
*Salmonella typhi**	0.00 ± 0.00 ^{a}	0.00 ± 0.00 ^{a}	12.33 ± 1.20 ^{b}	12.00 ± 0.58 ^{b}	11.33 ± 0.88 ^{b}	11.00 ± 0.58 ^{b}	ND
*Enterococcus faecalis**	1.50 ± 0.00 ^{a}	4.17 ± 0.17 ^{b}	11.33 ± 01.53 ^{c}	20.33 ± 0.58 ^{e}	15.33 ± 0.67 ^{d}	19.00 ± 1.53 ^{e}	ND
Escherichia coli ATCC 23718	6.00 ± 0.00 ^{b}	10.17 ± 0.17 ^{c}	10.33 ± 0.33 ^{c}	13.00 ± 0.58 ^{d}	0.00 ± 0.00 ^{a}	26.50 ± 0.29 ^{e}	ND
Escherichia coli ATCC 35218	7.33 ± 0.17 ^{b}	0.00 ± 0.00 ^{a}	15.00 ± 0.58 ^{c}	20.00 ± 0.58 ^{d}	24.00 ± 0.58 ^{e}	32.33 ± 0.88 ^{f}	ND
Pseudomonas aeruginosa ATCC 29853	0.00 ± 0.00 ^{a}	0.00 ± 0.00^{a}	11.00 ± 0.58 ^{b}	0.00 ± 0.00 ^{a}	0.00 ± 0.00 ^{a}	21.33 ± 0.88 ^{c}	ND
Bacillus cereus NCIB 6344	20.50 ± 0.29 ^{c}	0.00 ± 0.00 ^{a}	12.67 ± 0.88 ^{b}	14.33 ± 2.19 ^{b}	0.00 ± 0.00 ^{a}	25.50 ± 0.29 ^{d}	ND
Staphylococcus aureus NCIB 950	6.33 ± 0.17 ^{b}	0.00 ± 0.00 ^{a}	12.33 ± 1.20 ^{c}	11.33 ± 0.88 ^{c}	0.00 ± 0.00 ^{a}	14.83 ± 0.60 ^{d}	ND
Salmonella typhi ATCC 33458	12.17 ± 0.17 ^{a}	16.17 ± 0.17 ^{b}	12.67 ± 0.88 ^{a}	21.33 ± 0.88 ^{c}	21.00 ± 0.58 ^{c}	24.50 ± 0.29 ^{d}	ND
*Candida albicans**	0.00 ± 0.00 ^{a}	17.17 ± 0.17 ^{b}	ND	ND	ND	ND	15.35 ± 0.05 ^{c}
Aspergillus flavus	0.00 ± 0.00 ^{a}	0.00 ± 0.00 ^{a}	ND	ND	ND	ND	23.98 ± 0.017 ^{b}
Aspergillus fumigatus	0.00 ± 0.00 ^{a}	25.50 ± 0.29 ^{b}	ND	ND	ND	ND	32.33 ± 1.45 ^{c}

Keys: **TEA:** Acetone extract of *Trametes elegans*; **TEM**: Methanol extract of *Trametes elegans;* **MRSA**: Methicillin resistant *Staphylococcus aureus*; **ATCC**: American Type Culture Collection; **NCIB**: National Collection for Industrial Bacteria; *****: Clinical isolate; **ND**: Not determined.

Table 6. Minimum inhibitory concentration (mg/ml) of methanol and acetone extracts of *Trametes elegans* against test organisms.

Test Organism	TEM	TEA
Escherichia coli *	25	25
Pseudomonas aeruginosa *	50	25
*Enterococcus faecalis**	50	25
Escherichia coli ATCC 23718	50	12.5
Escherichia coli ATCC 35218	25	ND
Bacillus cereus NCIB 6344	12.5	ND
Staphylococcus aureus NCIB 950	12.5	ND
Salmonella typhi ATCC 33458	12.5	12.5
Candida albicans *	ND	12.5

Each value is mean of triplicate results.

Keys: **TEA:** Acetone extract of *Trametes elegans*; **TEM**: Methanol extract of *Trametes elegans*; *: Clinical strain; **ATCC**: American Type Culture Collection; **NCIB**: National Collection for Industrial Bacteria; **ND**: Not determined.

4. Discussion

Mushrooms are economically important since they serve as food, medicine, biocontrol agents, and a source of bioactive compounds used in the pharmaceutical and many other industries (Duarte *et al.*, 2006). Medicinal mushrooms have shown therapeutic benefits, primarily because they contain a number of biologically active compounds (Lee and Hong, 2011). The phytochemical properties and the antimicrobial potential of extracts of *T. elegans* indigenous to Nigeria were assessed in this study.

Species constituting the genus *Trametes* are similar in morphology; hence, the identification and separation of these species based on tradition taxonomy are difficult (Zhang *et al*., 2006). DNA sequences have been used in recent years to resolve the taxonomic problems in *Trametes* and in the related genera (Miettinen and Larsson, 2010; Cui *et al*., 2011). ITS sequences of the nuclear ribosomal DNA served as a useful molecular marker in distinguishing the species with similar morphological characteristics (Zhang *et al*., 2006; Cui *et al*., 2011).

In the present study, analysis of the ITS region of the nuclear rDNA revealed the genetic difference between the gene sequence of *Trametes* species collected from forest in Osengere, Ibadan Nigeria, and *Trametes* species sequences obtained from NCBI GenBank. The genes in DNA molecule are known to carry information that determines the characteristics of an organism. One of the implications in the difference between the genetic make-up of the indigenous *Trametes* species collected from Nigeria and the genes of their close relatives is that the type(s) and effectiveness of bioactives they produce may differ.

The differences in the ecological zones where these fungi exist and the influence of time might account for the difference in the gene sequence of *Trametes* species from Nigeria and its counterpart from other parts of the world. Wu *et al*. (2013), in their report, described the geographic distance as the dominant factor driving variation in fungal diversity at a regional scale (1000-4000 km), while environmental factors (total potassium and total nitrogen) account for the variation in fungal diversity at a local scale (<1000 km).

Results from the extraction process showed methanol giving a better yield than acetone. The high percentage yield of the methanol extract in this study may be due to the ability of the solvent to dissolve endogenous compounds (Anokwuru *et al*., 2011). Campos *et al*. (2002) in their findings also reported polar solvents to be more effective in extracting organic and inorganic materials from plants. Factors, such as the chemical nature of the compounds (simple and complex compounds), the extraction method employed, the extraction solvent, the extraction time and conditions such as temperature and pH of solvent, and the presence of interfering substances, can influence the extraction process (Brahmi *et al*., 2012). According to López *et al*. (2011), the nature of the solvent and the chemical properties of the sample are the two most important factors when extraction is subjected to similar conditions of time and temperature. This makes the processing efficiency quantitatively related to extraction yield (De Campos *et al*., 2008).

Results from the qualitative phytochemical analysis revealed the presence of saponins, tannins, steroids, terpenoids, flavonoids, and cardiac glycosides in all the *Trametes* species extracts, while alkaloids, anthraquinones and phlobatannins were absent. These phytochemicals are known to be biologically active, and serve as a defence mechanism for plants against predation by many microorganisms, insects and other herbivores (Bonjar *et al.*, 2004). This suggests that the mushroom can be used in the treatment of infectious diseases. For instance, Lim *et al*. (2006) reported on the antimicrobial activity of tannin extracted from *Rhizophora apiculata* bark. Saponins have been shown to demonstrate antimicrobial properties particularly against fungi, bacteria and protozoa (Sahelian, 2014). Taleb-Contini *et al*. (2003) reported the antibacterial activity of steroids isolated from *Chromolaena* species against *Streptococcus mutans* and *Streptococcus sobrinus* strains. Neumann *et al*. (2004) also confirmed the antiviral property of steroids.

Terpenoids have been reported to have anticarcinogenic (e.g. perilla alcohol), antimalarial (e.g., artemisinin), anti-ulcer, hepaticidal, antimicrobial or diuretic (e.g., glycyrrhizin) effects (Dudareva *et al*., 2004). Extracts of various medicinal plants containing phenolics and flavonoids have been reported to possess antimicrobial properties (Rahman and Moon, 2007; Ayaz *et al*., 2008). The variation observed in the amount of phytochemicals present could be as a result of the differences in the extraction capacity of the solvents used and the differences in the solubility of the different phytochemicals.

The extracts of *T. elegans* used in the present study displayed varying antimicrobial activities. This might be a result of a number of factors, as studies suggest that the antimicrobial activities of all mushroom extracts are changeable, depending upon the test organisms, nature of environment and media in which the test organism grows, genetic nature of the mushroom species, solvent used for extraction, and differences in physical and biochemical nature of the antimicrobial components of the mushroom extracts (Iwalokun *et al*., 2007; Ramesh *et al*., 2010). The differences in the antimicrobial activities of different species of mushrooms have been mainly attributed to the differences in the antimicrobial components found in them (Kosanic *et al*., 2013). Mushrooms require antibacterial and antifungal compounds in order to survive in their natural habitat (Lindequist *et al*., 2005). These attributes might make them a rich sources of natural antibiotics.

The variation observed between the Gram negative and Gram positive bacteria might be due to differences in their cell wall. Gram-negative bacteria have generally been reported to be more resistant to antimicrobials than Gram-positive bacteria. The outer membrane of Gram-negative bacteria plays an important role related to resistance to many antibiotics that are highly effective against Gram-positive bacteria, e.g., macrolides, novobiocin, rifamycin, lincomycin, clindamycin and fusidic acid (Sperandio *et al*., 2013). Gram-positive bacteria possess a porous layer of peptidoglycan and a single lipid bilayer, while Gram-negative bacteria have a double lipid bilayer sandwiching the peptidoglycan layer plus an outer layer of lipopolysaccharide, which results in a low degree of permeability for lipophilic small molecules (Sharma *et al*., 2011).

Results from the present study are, however, in contrast with the findings of several authors, who have reported the higher susceptibility of Gram positive bacteria than Gram negative bacteria. This implies that the antimicrobial activities of the extract of *T. elegans*, might not be cell wall related. The observed result in the present study is in line with the findings of Rakholiya *et al*. (2013) that observed higher susceptibility of Gram negative bacteria while studying the antimicrobial activity of decoction extracts of residual parts (seed and peels) of *Mangifera indica* L. var. Kesar against pathogenic and food spoilage microorganism.

The observed variation in the antibacterial activities of extracts of *T. elegans* to organisms with the same Gram reaction might be connected with their ability to produce capsule and slime. For instance, *E. coli*, *P. aeruginosa*

and *S. typhi* displayed different zones of inhibition. Being gram negative, the cell wall compositions are similar and the variation in their susceptibility might be capsular related. Another reason for the variation observed in organism with the same Gram reaction may be due to the presence of resistance factors like plasmids, transposons and insertion sequence (Clewell and Dunny, 2002; Nikaido, 2009). Plasmids are most commonly found in Gram negative bacteria and they often provide a selective advantage: many confer resistance to one or more antibiotics (Murray *et al*., 2013).

It was also observed that the clinical bacterial isolates and referenced bacterial cultures showed a marked difference in the zone of inhibition. The clinical bacterial isolates were found to be more resistant to the extracts than the typed bacterial cultures in most cases except for *P. aeruginosa* in which the referenced culture was more resistant than the clinical isolate. The resistance of the clinical isolates might be connected to the indiscriminate exposure of the clinical isolates to various antibiotics. Several clinical isolates have developed effective ways to deal with antibiotics through acquisition of resistant gene, production of enzymes such as the β-lactamases, changes in outer membrane porins that block the entry of the drug; and active pumping of the drug out of the cell using complex efflux pumps (Taiwo, 2011).

In contrast with the general trends that have reported a higher sensitivity of bacteria to antimicrobials than fungi, it was observed, in the present study, that the acetone extract of *T. elegans* exhibited higher antifungal than antibacterial activities. An earlier report by Pepeljnjak *et al*. (2005) also showed that fungi were more susceptible than bacteria while studying the antimicrobial activity of juniper berry essential oil. There has been an increase in the level of resistance of fungi to antifungal drug; hence, there is limited number of drugs available for the treatment of mycotic infections. Results from the present study, however, show that extracts of *T. elegans* may be an excellent source of antifungal drugs.

Although a number of natural/synthetic antimicrobial agents have been isolated/developed to control pathogenic microorganisms effectively, global antimicrobial resistance is still an increasing public health problem. Therefore, novel antimicrobial agents from different biological sources are continuously sought. Extracts of *T. elegans* used in the present study exhibited a varying degree of antibacterial and antifungal activities, and could be considered as potential sources of natural antimicrobials.

5. Conclusion

The present study was able to identify indigenous mushroom using molecular data, thus enriching and providing additional information on mushroom biodiversity in Nigeria. The study also showed the antimicrobial potentials of *T. elegans* collected in Osengere, Ibadan, thus giving credence to the therapeutic use of the mushrooms in folkloric medicine. Findings from this study are encouraging; however, further studies are required to isolate and characterize the specific biologically active agents responsible for the antimicrobial properties of the indigenous *T. elegans*. Also, preclinical and clinical studies are needed to establish the usefulness of the natural extracts of these mushrooms in the treatment or prevention of many human diseases.

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The Potential Allelopathic Effects of *Varthemia iphionoides* and the Identification of Phenolic Allelochemicals

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Abstract

The allelopathic effects of aqueous extracts from *Varthemia iphionoides* (Compositae) leaves were tested on germination and early seedling growth of wheat (*Triticum durum* Desf.), barley (*Hordeum vulgare* L.), chickpea (*Cicer arietinum* L.), lentil (*Lens culinaris* Medik.), tomato (*Solanum lycopersicum* L.), and pepper (*Capsicum annuum* L.). The experiments were conducted in petri dishes and treatments were arranged in a completely randomized design with eight replications. *V. iphionoides* aqueous extract was applied in 1.25, 2.5, 5.0 and 10% concentrations and water was used as a control. Germination was observed daily and, after 7 days, radicals length and seedlings dry weight were recorded. Total germination percentage, germination rate, seedling vigor index, and seedling dry weights were significantly inhibited by *V. iphionoides* aqueous extracts. The germination percentage of chickpea seeds was almost unaffected by the treatment. The extract enhanced germination and seedling dry weights in chickpea at the lowest extract concentration. Tomato and wheat were the most sensitive to *V. iphionoides* extract followed by pepper and barley. On the other hand, chickpea and lentil were less affected. Using LC-ESI-MS/MS analysis, ten phenolic compounds were identified in *V. iphionoides* leaves. The results of the current study suggested that *V. iphionoides* aqueous extract possesses allelopathic properties and extract activities are species-specific and concentration-dependent.

Keywords: Allelopathy, germination, phenolic compounds, seedling vigor index, *Varthemia iphionoides*, aquous extracts.

1. Introduction

Allelopathy is defined as any direct or indirect harmful or beneficial effects of one plant species on another through the release of phytochemicals (allelochemicals) under natural conditions (Rice, 1984). Allelochemicals are present in almost all plant organs and are released into the environment via a variety of processes, including root exudation, volatilization, leaching, and decomposition of plant residues (Inderjit and Duke, 2003; Weston and Duke, 2003).

The phenylpropanoid pathway is the main source of allelochemicals in plants. These allelochemicals are diverse in structures and mode of actions and are known to inhibit growth, development, and several physiological processes in receptor plants (Gniazdowska and Bogatek, 2005; Lorenzo *et al*., 2008); in consequences, affecting competition and dominance within plant communities and contribute to the success of invasive species (Abu-Romman and Ammari, 2015; Callaway and Ridenour, 2004; Ens *et al*., 2009). Some plant species are able to inhibit their own kind through the release of phytotoxic compounds (Singh *et al*., 2009). Allelopathy is currently practiced in organic agriculture as a biological and eco-friendly practice of weed control instead of using herbicides (Hoagland *et al*., 2008; Khaliq *et al*., 2010; Vyvyan, 2002).

Varthemia iphionoides Boiss is a member of the Compositae family and is widely distributed in Jordan mainly in semidry lands (Al-Eisawi, 1982). *V. iphionoides* is a 30-80 cm long bushy-perennial herb with many aromatic and sticky stems (Feinbrun-Dothan, 1977). Aqueous extracts of *V. iphionoides* are used in folk medicine of the eastern Mediterranean region as infusion against diabetes mellitus and gastrointestinal disorder (Afifi *et al*., 1997). Furthermore, flavonoids isolated from this medicinal plant exhibited antimicrobial activity and

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* **Abbreviations**: GR: germination rate; LC-ESI-MS/Ms.: liquid chromatography-electrospray ionization-tandem mass spectrometry; LSD: least significant difference; SVI: seedling vigor index.

antiplatelet activity on human blood (Afifi *et al*., 1991; Afifi and Aburjai, 2004; Al-Dabbas *et al*., 2006).

V. iphionoides has become a noxious weed in field crops in Jordan and inhibition zones around this plant in the fields were noticed (Abu-Romman, personal observations). Therefore, it would be reasonable to propose that *V. iphionoides* plants exhibit allelopathic effects. Hence, this study was designed to determine whether aqueous extracts of *V. iphionoides* leaves exhibit allelopathic potential on germination characteristic and early seedling growth of wheat, barley, chickpea, lentil, tomato, and pepper. In addition, we identified phenolic compounds in *V. iphionoides* leaves using LC-ESI-MS/MS analysis.

2. Materials and Methods

2.1. Plant material and extract preparation

Leaves of *Varthemia iphionoides* were collected in August, 2013 from wild population in Al-Salt, Jordan (32º12'N latitude; 35º74'E longitude; 800 m above sea level). Leaves were well cleaned with tap water. Leaf tissues were oven-dried at 50 °C for three days and ground to a fine powder.

A hundred grams of tissue powder were used to make aqueous extract by soaking in 1 L of distilled water for 24 h at room temperature. The resultant extract was first filtered through three layers of cheesecloth to remove tissue debris, followed by a second filtration through Whatmann No. 1 filter paper. The extracts obtained were considered to be the stock extract (10 % w/v). The stock extract was diluted to give final concentrations of 1.25, 2.50, 5.00 and 10 % (w/v). Distilled water was used as a control.

2.2. Bioassay

The agricultural crops selected were wheat (*Triticum durum* Desf.), barley (*Hordeum vulgare* L.), chickpea (*Cicer arietinum* L.), lentil (*Lens culinaris* Medik.), tomato (*Solanum lycopersicum* L.), and pepper (*Capsicum annuum* L.). All seeds were surface-sterilized in 70% ethanol for 10 min and then by soaking in 2% sodium hypochlorite for 5 min followed by rinsing several times in sterile water.

Fifteen seeds of each species were evenly placed on three-layer filter papers (Whatmann No. 1) in a sterilized 9-cm Petri dishes. All Petri dishes were placed in a dark incubator at 25 ± 1 °C.

Initially, filter papers were moistened with 10.0 ml of respective extract concentration as treatment and distilled water as control. Two ml of the extract solution for the treatment and 2.0 ml of distilled water for the control were added daily. Germination was observed daily over a 7-day period. Germination was considered to occur only after the radical had protruded beyond the seed coat by at least 1.0 mm. Seven days after sowing, the germination percentage was calculated using the formula: [(germinated seeds/total seeds) × 100]. The rate of germination (GR) was calculated according to the formula of Noor *et al*. (1995): [$\Sigma G/t$], where G is the number of seeds germinated at every day and t is the total germination period in days.

Seedling Vigor Index (SVI) was calculated using the formula: [germination % × radical length (cm)] (Abdul-baki and Anderson, 1973). Dry weight of seedlings were measured 7 days after germination by drying seedlings in an oven at 50 °C until the seedlings are completely dry and their weights are constant.

2.3. Identification of phenolic allelochemicals

Identification and quantification of phenolic allelochemicals were performed using liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS).

The analysis of phenolic compounds in *V. iphionoides* extract was carried out using Agilent 1100 chromatography system (Agilent 1100, Agilent Technologies, Wilmington, DE, USA) equipped with a diode array UV detector. The samples were injected into a Thermo C_{18} reversed phase column (pore size 5 µm, 250× 4.6 mm i.d. Thermo Fisher Scientific, San Jose, CA, USA).

The analysis employed a gradient solvent system using aqueous formic acid (1%) as solvent (A), and methanol/acetonitrile/formic acid mixture (89.5/9.5/1 v/v/v) as solvent (B). A seven-step linear gradient elution for a total run time of 65 min was carried out using the solvent gradient as follows: 0-10 min 90-70 solvent A and 10-30% solvent B, 10-15 min isocratic, 15-25 min 60 solvent A and 40% solvent B, 25-40 min 50% solvent A and 50% solvent B,40-50 min to 100% solvent B, 50-55 min 90% solvent A and 10% solvent B and 55-65 min isocratic. An injection volume of 15 µL at constant flow of 0.75 mL/min was employed. The entire flow from the HPLC was directed into the triple-quadrupole mass spectrometer (API 3200; MDS Sciex, Concord, ON, Canada). The mass spectral data were acquired on a negative ion mode. The ESI conditions were as follows: capillary voltage of 4000 V, the ion source was Atmospheric Pressure Chemical Ionization, a cone voltage of 70 V. The dry temperature was 350°C and the drying gas, N_2, had a flow rate of 4.0 L/min. Product ion scans for mass were performed by low-energy collision (10 eV) and helium was used as the nebulizer gas with a flow rate of 40 psi. Diode array UV detector was used to scan mode between 200 and 400 nm to evaluate contents of individual phenolic compounds. A maximum absorbance of 280 nm was selected to determine the contents of the individual phenolic compounds. The mixture of external standards was used to quantify the contents of individual phenolic compounds. All LC-ESI-MS/MS data were processed by Bruker Daltonics data analysis software (version 4.0).

2.4. Statistical analysis

The experiment was designed to have eight replications using a completely randomized design. All values were expressed as percentages compared to the control. Means are separated using the LSD test, and statistical significance was evaluated at $P < 0.05$.

3. Results

The effects of aqueous extract at different concentrations from *V. iphionoides* on germination percentage of six test plant species are shown in Figure 1. The results illustrated that germination percentage of all test species except for chickpea was significantly altered. The response toward the inhibitory potential of the aqueous extracts varied among the examined species. At all concentrations of aqueous extract; the final germination percentages of wheat, barley, and tomato were significantly reduced with each increase in the concentration of *V. iphionoides* aqueous extracts. Pepper germination percentage was only significantly reduced at 5% and 10% extract concentrations. The final germination percentage of chickpea was not significantly altered in response to *V. iphionoides* aqueous extracts. The lentil germination percentage was significantly reduced at only 5% and 10% extract concentration (Figure 1).

The application of *V. iphionoides* aqueous extracts had significant effects on the germination rate (GR) of all the examined species (Figure 2). GRs of wheat, barley, tomato, and pepper were significantly suppressed by all applied concentrations of the extract. At 10% extract concentration, the GRs relative to the control were 55.7%, 46.4% and 54.8% for wheat, tomato and pepper, respectively.

An enhancement in GR compared to the control was recorded for chickpea when treated with 1.25% extract concentration. The GR of chickpea was only significantly reduced at the highest extract concentration (10%) that was used in the experiment. GR of lentil had the lowest values at 10% extract concentrations. However, lentil and chickpea GRs were least inhibited regardless of the aqueous extract concentrations compared to wheat, barley, tomato, and pepper.

The Seedling Vigor Index (SVI) of all the examined species was significantly inhibited in response to *V. iphionoides* aqueous extract treatment (Figure 3). SVI recorded the lowest values in tomato and wheat followed by pepper and barley. On the other hand, SVI of lentil and chickpea was only significantly lowered by the application of the highest extract concentration (10%). It was noted that the lowest concentration (1.25%) of aqueous extract stimulated chickpea SVI compared to the control. Tissue browning in the radical of tomato seedlings was noticed at 5 and 10% concentrations of *V. iphionoides* aqueous extract (data not shown).

The seedling dry weights were also significantly affected in response to the aqueous extracts of *V. iphionoides* (Figure 4). Among all examined crops, the dry weights of tomato and wheat seedlings were the most negatively affected, followed by pepper and barley. The dry weight of lentil seedlings was less affected compared to the previously mentioned crops. A slight increase in the dry weight of chickpea seedlings was observed at 1.25% extract concentration. However, the dry weight of chickpea seedlings was only reduced significantly when treated with the highest aqueous extract concentration.

The determination of phenolic compounds in *V. iphionoides* leaf extract was performed using LC-ESI-MS/MS analysis. The results presented in Table 1 revealed the presence of ten phenolic compounds in *V. iphionoides* leaves, i. e. *p*-hydroxybenzoic acid, ferulic acid, vanillic acid, tyrosol, chlorogenic acid, *p*-coumaric acid, naringin, quercetin, sinapic acid and rosmarinic acids. Among these phenolic compounds, vanillic acid was the most prominent in leaves of *V. iphionoides*, followed by *p*-coumaric acid, tyrosol and ferulic acid.

Table 1. The nature and concentration of phenolic compounds identified by LC-ESI-MS/MS analysis from leaves of *V. iphionoides*.

Phenolic compound	Concentration (µg g^{-1} dry eaves)
p-Hydroxybenzoic acid	2.4
Ferulic acid	28.8
Vanillic acid	139.6
Tyrosol	36.4
Chlorogenic acid	7.94
p-Coumaric acid	47.2
Naringin	0.104
Quercetin	1.42
Sinapic acid	2.88
Rosmarinic acids	4.36

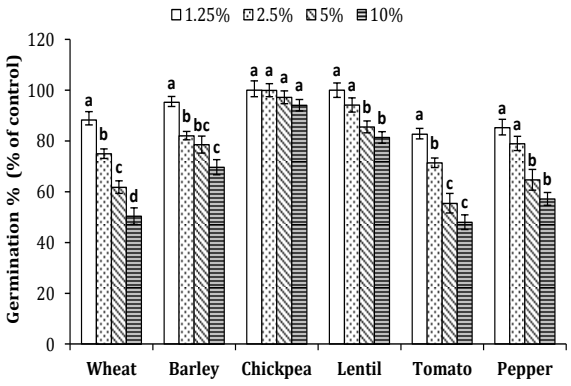

Figure 1. Effect of aqueous extract at different concentrations from *V. iphionoides* leaves on final germination percentages of six agricultural crops. Results are presented as the percentage of control plants. Different letters within the same crop species indicate significant differences using LSD test at $P < 0.05$.

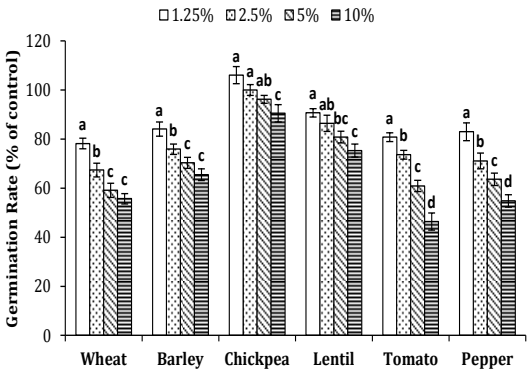

Figure 2 .Effect of aqueous extract at different concentrations from *V. iphionoides* leaves on germination rates of six agricultural crops. Results are presented as the percentage of control plants. Different letters within the same crop species indicate significant differences using LSD test at $P < 0.05$.

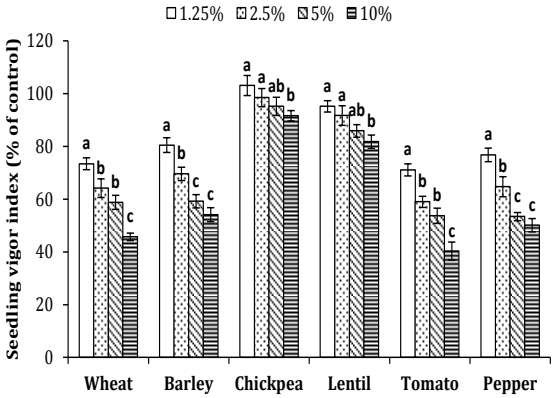

Figure 3. Effect of aqueous extract at different concentrations from *V. iphionoides* leaves on seedling vigor indexes of six agricultural crops. Results are presented as the percentage of control plants. Different letters within the same crop species indicate significant differences using LSD test at $P < 0.05$.

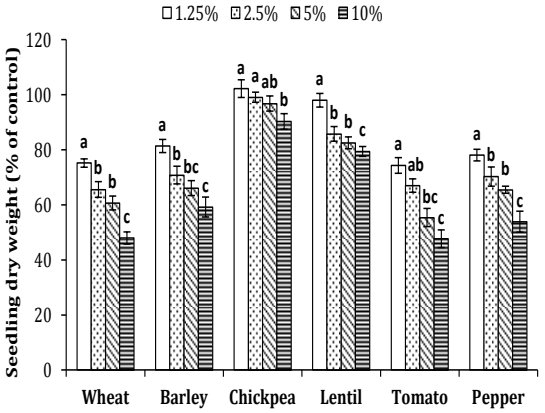

Figure 4.Effect of aqueous extract at different concentrations from V. iphionoides leaves on seedling dry weights of six agricultural crops. Results are presented as the percentage of control plants. Different letters within the same crop species indicate significant differences using LSD test at $P < 0.05$.

4. Discussion

The present results indicate that aqueous extracts of *V. iphionoides* plants contain indeed growth inhibitors that are capable of reducing germination and growth of the studied species. The responses of the studied species to the aqueous extracts of *V. iphionoides* were markedly different. This indicates that the degree of allelopathic interference of *V. iphionoides* aqueous extracts were quite species-specific. Similar results were previously reported with different target species and allelochemical sources (Bhowmik and Inderjit, 2003; El Ayeb *et al*., 2013; Mutlu and Atici, 2009; Vrchotová *et al*., 2011). The different responses among species and even within species to allelochemicals result mainly from the target plant genetic makeup, which is responsible for the physiological and the biochemical characteristics (Kobayashi, 2004; Prati and Bossdorf, 2004). Moreover, the differential variation in the target species responses to allelochemicals could result in part to the biometric parameters of the plant seeds (Pellissier, 2013).

With exception of chickpea, final germination percentages of the test species were reduced and were proportional to the extract concentrations applied. Moreover, the germination process was delayed in response to the treatment with *V. iphionoides* aqueous extract. However, treating chickpea seeds with the lowest extract concentration showed slight enhancement of GR. The effect of aqueous extracts on SVI were almost similar to those observed in GR.

Allelochemicals are known to influence the metabolic and physiological processes during seed germination (Abu-Romman, 2011; Chon *et al*., 2005; Rashid *et al*., 2010). Phenolic allelochemicals were shown to delay seed imbibition and inhibit germination enzymes, which, therefore, resulted in slowing down starch degradation and sucrose hydrolysis during germination (Lara-Núñez *et al*., 2009; Politycka and Gmerek, 2008; Singh *et al*., 2009; Tawaha and Turk, 2003).

Stimulation of GR and SVI by lower concentration of the extract was previously reported (Duke *et al*., 2006; Liu and Chen, 2011; Sampietro and Vattuone, 2006; Singh *et al*., 2008). Lower concentrations of allelochemicals were shown to stimulate amylase activity and therefore increased soluablization of starch during the germination process of some species (Rizvi *et al*., 1989; Singh *et al*., 2009).

Seedling dry weights were negatively affected by treatments with aqueous extracts of *V. iphionoides*. Allelochemicals were shown to impose oxidative stress on the target species. This oxidative stress interferes with cell division, cell elongation, and phytohormone induced growth (Golisz *et al*., 2008; Jacob and Sarada, 2012; Nishida *et al*., 2005).

The legumes examined in the present study were found relatively resistant to the allelopathic *V. iphionoides* plant. Some plant species possessed detoxification mechanisms to cope with allelochemicals (Weir *et al*., 2004). These detoxification mechanisms include the oxidation, hydroxylation or glucosylation of the phytotoxic compounds (Inderjit and Duke, 2003; Sicker *et al*., 2001; von Rad *et al*., 2001).

Phenolics are the most important and common allelochemicals known to play a significant role in the ecosystem (Batish *et al*., 2007; Li *et al*., 2010). The phytotoxicity of phenolic allelochemicals was reported as a result of their ability to disrupt normal metabolic processes in the plant (Weir *et al*., 2004). Via LC-ESI-MS/MS analysis, ten phenolic compounds with varied concentrations were detected in *V. iphionoides* leaves. Among them, vanillic acid, *p*-coumaric acid, tyrosol and ferulic acid were dominant (Table 1). *V. iphionoides* plants were reported to contain remarkably high levels of total phenolic contents compared to other medicinal plants within the same Compositae family (Alali *et al*., 2007). The allelopathic impacts of some phenolic compounds identified in the present study were previously documented. Blum and Gerig (2005) reported that vanillic, *p*-coumaric and ferulic acid inhibited transpiration, water utilization, leaf area expansion and dry weight of cucumber seedlings. These phenolic acids were also reported to reduce chlorophyll content of soybean plants (Patterson, 1981). Moreover, ferulic and *p*-

coumaric acids were shown to enhance lipid peroxidation and modulate antioxidant system in maize and soybean seedlings (Devi and Prasad, 1996; Doblinski *et al*., 2003).

5. Conclusion

In conclusion, *V. iphionoides* showed an allelopathic potential on the crop plants under investigation. The effects of allelochemicals produced in this plant are both species-specific and concentration-dependent. The magnitude of reduction in seed germination and seedling dry weights after treatment with *V. iphionoides* aqueous extracts followed the following order: tomato > wheat > pepper > barley > lentil > chickpea. These findings are valuable for avoiding the cultivation of some crop species (e.g., tomato and wheat) in fields infested with *V. iphionoides* plants. LC-ESI-MS/MS analysis revealed the presence of several phenolic compounds in *V. iphionoides* leaves that possibly contribute to the observed phytotoxicity of this medicinal plant. The present results are obtained under laboratory conditions. Laboratory bioassays were commonly used as a first step toward exploring the potential allelopathic effects of a certain plant species (Inderjit and Callaway, 2003). However, field studies are still needed to test the allelopathic properties of *V. iphionoides* under more natural conditions. Further studies on isolation, purification and evaluation of the candidate allelochemicals present in *V. iphionoides* are required to gain a better understanding of its physiological and biochemical mechanisms of action.

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Identifying Selection Signatures Related to Domestication Process in Barley (*Hordeum vulgare* L.) Landraces of Jordan Using Microsatellite Markers

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Abstract

Domestication process and the subsequent breeding may result in a genetic mutation and selection pressures that possibly contributed to the emergence of two phenotypes of barley spikelets, namely six-row and two-row barley. The present study used microsatellite genetic markers to investigate the population genetics of a selected set of accessions of domesticated barley representing different populations of Jordan, to identify a signature of selection resulted from domestication process. Genomic and statistical approaches, such as the analysis of molecular variance (AMOVA) and the so-called hierarchical "outlier" tests, were utilized to identify signatures of selection. Generally, there was a high level of polymorphisms in all the studied populations, which ranged from 19.23 for Ramtha to 69.23 (%) for Zarga, with an average of 53.61 (%). The average number of allele per locus for the entire sample set was of 9.346 and it ranged 2 occurring at [(Bmag0136), (EBmac0970_a), (EBmac0970_c) loci], to 27 at (Bmac0040) locus. The results of AMOVA showed that the main portion (~72%) of total diversity was attributed to differentiations within populations. Other portions of diversity (11.040%) were explained by the diversity among populations. As revealed by the hierarchical outlier tests and AMOVA results, 9 microsatellites were identified to be under possible selection pressures, possibly indicates that these loci were important in the past improvement of barley by early cultivators. In conclusion, the present study shows that barley landraces of Jordan possess high levels of genetic diversity and allelic richness that could be utilized in barley improvement and breeding program.

Keywords: Hordeum spp, microsatellite, genetic diversity, population differentiation, domestication, selection signature, Jordan.

1. Introduction

Modern cereals, such as barley (*Hordeum vulgare* L.), have been originated as a result of the domestication process which is believed to be started ~12,000 years before present (yBP) in the Fertile Crescent, a region which encloses the countries of Iraq, Kuwait, Syria, Lebanon, Jordan, Palestine, Cyprus, and Egypt, southeastern border of Turkey, and the western border of Iran (Zohary and Hopf, 2000; Salamini *et al*., 2002). This domestication process and the subsequent selective breeding by early cultivators of cereals involved the selection of individual's plants with certain traits that serve human needs, such as improved seed yield, seed recovery, and other traits (Hammer, 1984; Harlan, 1992). In barley, the selection process by humans during domestication resulted in the emergence of two different phenotypes or morphs, namely six-rowed barley or (*Hordeum vulgare* L. *hexastichum*), and two-rowed barley or (*Hordeum vulgare* L. *distichum*) (Badr *et al*., 2000; Kilian *et al*., 2006). Population genetics studies indicated that during domestication process the six-rowed barley has emerged from the two-rowed wild barley as a result of mutations and selection pressures which lead to the difference in morphology of spikelets (Komatsuda *et al*., 2007).

Studying population genetics of domesticated crops, such as barley, is important not only from an evolutionary stand point, but also from agricultural perspectives (Meyer and Purugganan, 2013; Fuller *et al*., 2011; Kellogg, 2001). For example, an efficient breeding

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method requires a selection of agriculturally important phenotypes which are often genetically controlled by an array of genotypic spectrum (Fuller *et al*., 2011).

Microsatellites, also called Simple Sequence Repeats (SSRs), are among the advanced genetic markers currently developed (Struss and Plieske, 1998). These genetic markers are considered to be highly informative and reproducible in term of their discrimination among populations and landraces of barley (Ramsay *et al*., 2000)

In the present study, the population genetics of a selected set of accessions of domesticated barley covering ten populations (sites) of Jordan were investigated using microsatellite genetic markers. Specifically, the objectives of this study are to: (1) investigate the level of genetic diversity within and between populations of barley, (2) study the degree of population differentiation between the two cultivated phenotypes (two-rowed and six-rowed barley) and (3) identify microsatellite loci that are under possible influence by selection pressure of domestication.

2. Materials and Methods

2.1. Plant Material and Genomic DNA Isolation

A set of 139 seeds representing accessions of barley landraces (*Hordeum vulgare* L.), originally collected from 10 geographical locations of Jordan, were obtained from the seed bank of the National Center for Agricultural Research and Extension (NCARE, Weltzien, 1988). Of these accessions, 42 were of six-rowed spike barley type and 97 of two-rowed barley spike type. Seeds were grown in greenhouse and genomic DNA was extracted from young leaves of about 4 weeks using the CTAB method (Doyle and Doyle, 1987). An average of ten seeds (individuals) per site were used in the present study. Most seeds present in NCARE are of two-row compared with six-row barley.

2.2. Genotyping of microsatellite markers

Twenty-five microsatellite loci covering the 7 chromosomes of barley were chosen based on the genomic map established by Ramsay *et al*., (2000) (Table 1). Polymerase Chain Reactions (PCR) were carried out in a volume of about 12 µl containing approximately 20 ng of genomic DNA template, 0.5 U Taq polymerase (Qiagen, Hilden, Germany), 1 x PCR reaction buffer (Qiagen), 0.5 pmol each of forward and reverse primers (IRD700- or IRD 800-labelled), and 0.18 mM of each deoxynucleoside triphosphates (dNTPs). Amplifications were performed using a standard amplification cycle in a GeneAmp PCR System 9700 thermal cycler, and SSR polymorphisms were resolved and visualized using a LI-COR GeneReadir 4200 (MWG Biotech, Ebersberg). DNA isolation, PCR conditions, genotyping of microsatellite and gel preparation detailed in (Hasan *et al*., 2006). The laboratories techniques for this were conducted in the laboratories of the Justus Liebig University-Giessen/Germany.

2.3. Analysis of the level of genetic diversity

Summary statistics of genetic diversity, including gene diversity (Nei, 1978), the number polymorphic loci, percentage of polymorphism, and alleles number, were calculated for each microsatellite locus per population using the software Powermaker version 3.25 (Liu and Muse, 2005).

2.4. Identifying selection signature at microsatellite loci in relation to domestication

In order to detect and identify microsatellite loci that were possibly under selection pressure due to domestication, F_{ST}-based outlier test was conducted. This method takes hierarchical population structure into account (Excoffier *et al*., 2009), and is implemented in the software Arlequin. Briefly, the method detects outliers exhibiting significantly high or low F_{ST} values, controlled for heterozygosity at microsatellite loci. Outliers are detected based on differentiation among all populations (F_{ST}) and differentiation among groups of populations (two- rowed barley vs. six-rowed barley) (F_{CT}). The analyses were conducted at: (1) an overall analysis encompassing all analyzed populations (based on F_{ST}) and (2) an analysis at the phenotypic level conducting tests for pair-wise comparisons (F_{CT}). This analysis assumed a simulation model of 25 groups each consisting of 50 populations and based the tests on 50,000 simulated loci for the full data set.

3. Results

The 25 microsatellite loci used in the present study provided a fair coverage of the genome, with a range between 2 and 5 loci for each of the seven *Hordeum* linkage groups (Chromosomes). The final dataset, therefore, was based on results of 229 amplified SSRs with an average of 9.16 alleles per locus in the entire sample. The results show a large variation in the number of alleles among the microsatellite loci, with the lowest of 2 occurring at the (Bmag0136), (EBmac0970_a), and (EBmac0970_c) loci and the highest of 27 at (Bmac0040) locus. Nine of the (25) loci showed more than 10 alleles per locus (Table 1).

To see the level and the extent of genetic diversity within our studied samples of barley, summary statistics of genetic diversity were estimated using several parameters for each populations of barley. The average gene diversity for the entire samples set was found to be 0.468, with a range of 0.096 for Ramtha population to 0.610 for Zarga population (Table 2). Generally, there was a high level of polymorphisms in all the studied populations, which ranged from [19.23 for Ramtha to 69.23 (for Zarga)] with an average of 53.61 %.

To see the level of the population genetic differentiation that might reflect geographical or phenotypical structure between barley landraces, the total genetic diversity was divided within and between-populations components by using the analysis of molecular variances (AMOVA). The results of AMOVA show that the main portion of the total diversity of 71.830% was attributed to differentiations within populations (Table 3). Other portions of diversity (11.040%) were explained by the diversity among populations within phenotype types and (17.130%) percentage of variances was clarified by differentiation between the barley phenotype types, namely two-rowed and six-rowed barley. The high F_{CT} value of 17.13%,

which measures the extent of differences between barley phenotypes, indicated that genetic differentiation between (*Hordeum vulgare* L. *hexastichum*), and two-rowed barley (*Hordeum vulgare* L. *distichum*) is a considerably high. There was no significant correlation between geographic origins and genetic differentiation of barley accessions. As suggested by the hierarchical outlier test, it was found that 8 microsatellite loci to be under selection across all populations (F_{ST}; Figure 1), whereas 9 outliers microsatellite loci were identified to show selection pressures which contributed to the phenotypic characteristics of studied accessions of barley (F_{CT}; Figure 2).

Table 1. Marker name, allele number, chromosome location, primer pairs sequences, and repeat motif for 25 microsatellite markers used in this study.

Marker or Loci	Allele number	Chromosome	Primer sequence (5′–3′)	Repeat motif
Bmag0382	7	1H	5' TGAAACCCATAGAGAGTGAGC 3' 5' TCAAAAGTTTCGTTCCAAATAC 3'	(AG)7AA(AG)7
Bmag0211	10	1H	5' ATTCATCGATCTTGTATTAGTCC 3' 5' ACATCATGTCGATCAAAGC 3'	(CT)16
Bmag0347	14	1H	5' CTGGGATTGGATCACTCTAA 3' 5' AAAACAAGTACTGAAAATAGGAGA 3'	(CT)28
Bmac0093	5	2H	5' CGTTTGGGACGTATCAAT 3' 5' GGGAGTCTTGAGCCTACTG 3'	(AC)24
EBmac0850	5	2H	5' CTCAGATAACACCTTTAAACACA 3' 5' AAGACAGTTGGGTAAGCCT 3'	(AC)13AT(AC)10A-(AC)5
Bmac0134	21	2H	5' CCAACTGAGTCGATCTCG 3' 5' CTTCGTTGCTTCTCTACCTT 3'	(AC)28
Bmag0136	2	3H	5' GTACGCTTTCAAACCTGG 3' 5' GTAGGAGGAAGAATAAGGAGG 3'	(AG)6-(AG)10-(AG)6
HVM60	10	3H	5' CAATGATGCGGTGAACTTTG 3' 5' CCTCGGATCTATGGGTCCTT 3'	(AG)11 and (GA)14
Bmac0181	9	4H	5' ATAGATCACCAAGTGAACCAC 3' 5' GGTTATCACTGAGGCAAATAC 3'	(AC)20
Bmag0353	12	4H	5' ACTAGTACCCACTATGCACGA 3' 5' ACGTTCATTAAAATCACAACTG 3'	(AG)21
HVM67	8	4H	5' GTCGGGCTCCATTGCTCT 3' 5' CCGGTACCCAGTGACGAC 3'	(GA)11
Bmag0384	7	4H	5' TGTGAGTAGTTCACCATAGACC 3' 5' TGCCATTATCATTGTATTGAA 3'	(AG)18
Bmag0337	11	5H	5' ACAAAGAGGGAGTAGTACGC 3' 5' GACCCATGATATATGAAGATCA 3'	(AG)22
Bmac0163	4	5H	5' TTTCCAACAGAGGGTATTTACG 3' 5' GCAAAGCCCATGATACATACA 3'	(AC)6(GC)3(AC)17
EBmac0970_a	2	5H	5' ACATGTGATACCAAGGCAC 3' 5' TGCATAGATGATGTGCTTG 3'	(AC)8
EBmac0970_b	3	5H	5' ACATGTGATACCAAGGCAC 3' 5' TGCATAGATGATGTGCTTG 3'	(AC)8
EBmac0970_c	2	5H	5' ACATGTGATACCAAGGCAC 3' 5' TGCATAGATGATGTGCTTG 3'	(AC)8
Bmac0096	15	5H	5' GCTATGGCGTACTATGTATGGTTG 3' 5' TCACGATGAGGTATGATCAAAGA 3'	(AT)6(AC)16

Bmag0009	7	6H	5' AAGTGAAGCAAGCAAACAAACA 3' 5' ATCCTTCCATATTTTGATTAGGCA 3'	(AG)13
Bmac0040	27	6H	5' ATTATCTCCTGCAACAACCTA 3' 5' CTCCGGAACTACGACAAG 3'	(AC)20
Bmag0321	9	7H	5' ATTATCTCCTGCAACAACCTA 3' 5' CTCCGGAACTACGACAAG 3'	(AG)17(AC)16
Bmac0273_a	9	7H	5' ACAAAGCTCGTGGTACGT 3' 5' AGGGAGTATTTCACCCTTG 3'	(AC)20(AG)20
Bmac0273_b	5	7H	5' ACAAAGCTCGTGGTACGT 3' 5' AGGGAGTATTTCACCCTTG 3'	(AC)20(AG)20
Bmac0031	9	7H	5' AGAGAAAGAGAAATGTCACCA 3' 5' ATACATCCATGTGAGGGC 3'	(AC)28
Bmag0120	16	7H	5' AGAGAAAGAGAAATGTCACCA 3' 5' ATACATCCATGTGAGGGC 3'	(AC)28

Table 2. Summary statistics of genetic variation estimated for the studied populations of Jordanian landraces of barley.

Population/row-type	Sample size	Gene Diversity	No of Polymorphic loci	% of Polymorphic loci
Irbid/Two-rowed	11	0.555	16	61.538
Irbid/Six-rowed	22	0.588	11	42.308
Ramtha/Two-rowed	3	0.096	5	19.231
Jarash/Two-rowed	4	0.366	16	61.538
Mafrag/Two-rowed	8	0.572	12	46.154
Mafrag/Six-rowed	6	0.474	16	61.538
Zarga/Two-rowed	7	0.559	18	69.231
Zarga/Six-rowed	11	0.61	16	61.538
Balga/Two-rowed	2	0.558	14	53.846
Balga/Six-rowed	6	0.383	17	65.385
Madaba/Two-rowed	9	0.475	16	61.538
Madaba/Six-rowed	5	0.491	18	69.231
Karak/Two-rowed	18	0.564	13	50
Karak/Six-rowed	3	0.348	13	50
Tafileh/Two-rowed	16	0.347	14	53.846
Maen/Two-rowed	14	0.498	8	30.769

Table 3. AMOVA of barley populations investigated in this study showing the effect barley inflorescence types (two vs six row) on genetic differentiation.

Source of variation	df	Sum of squares	Variance	% variation	P value
Among genome types	1	99.138	0.777	17.130	P<0.001
Among populations within genotype types	14	150.217	0.501	11.040	P<0.001
Within populations	232	756.693	3.262	71.830	
Total	247	1006.048	4.541	100	

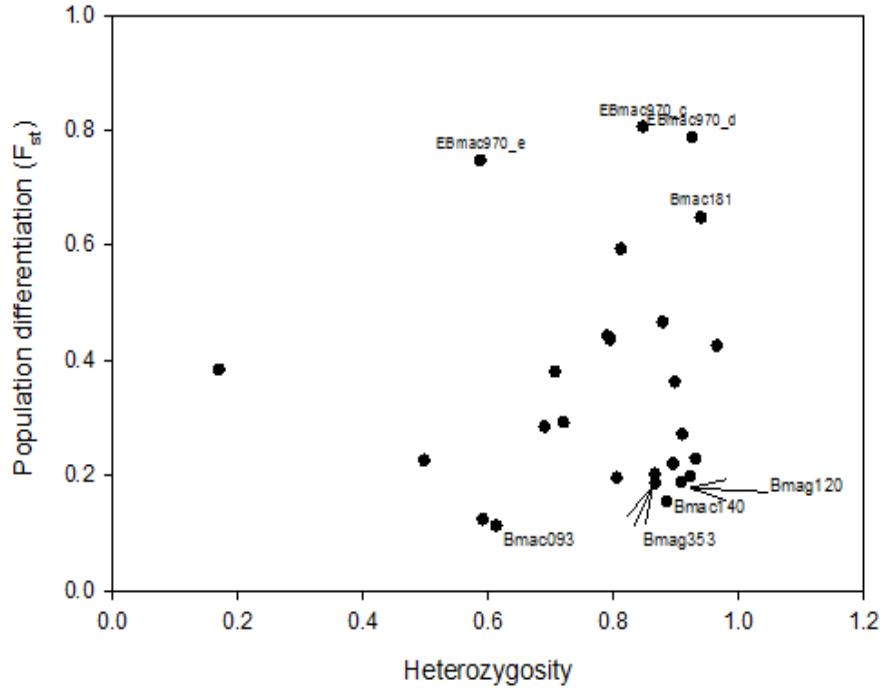

Figure 1. Graphical representation of the relationship between heterozygosity and population differentiation as obtained from hierarchical outlier tests involving the 25 microsatellites in this study. The graph shows the eight loci which are supposed to be under selection based on genetic differentiation between the two barley phenotypes.

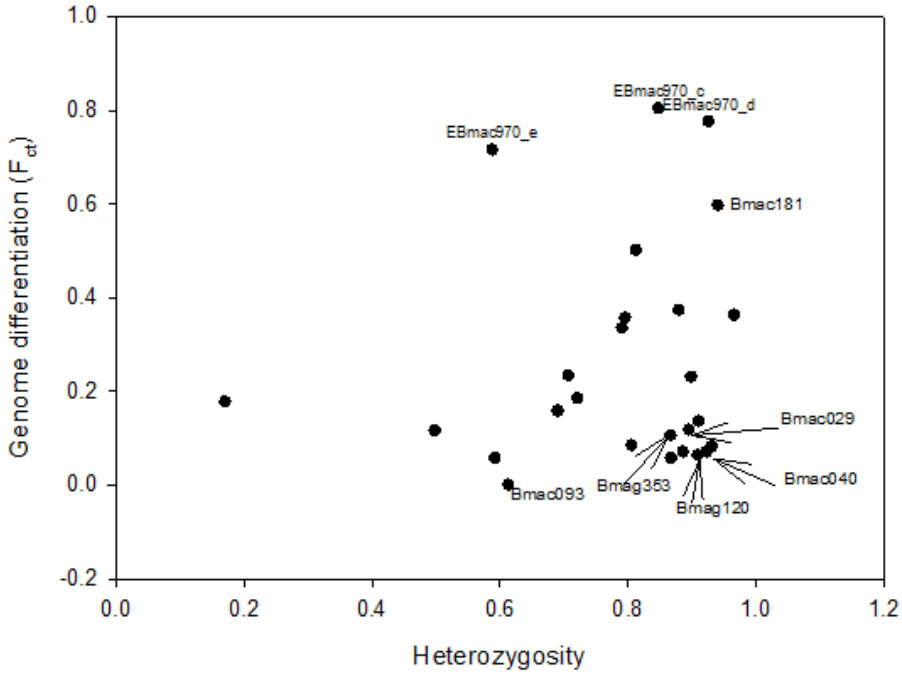

Figure 2. The relationship between heterozygosity and population differentiation as obtained from hierarchical outlier test based on genetic differentiation between the two barley phenotypes indicating the 9 microsatellite loci that show signature of selection pressures of barley domestication.

4. Discussion

With the advances of molecular markers, population genetics of crop plant has become an important tool for conserving and maintaining germplasm collections. Studies of population genetics of crops offer a unique opportunity to identify footprints or signature of selection which can give valuable insight which help to identify new genes of agronomic importance (Vigouroux *et al*., 2002; Burger, 2008; Fuller *et al*., 2011). Similar to other studies, the present study employed microsatellite, as suitable molecular markers to study the population genetic diversity in barley landraces (Ordon *et al*., 1995; Thiel *et al*., 2003; Varshney *et al*., 2006). This study of barley revealed a relatively high overall genetic diversity for all

the populations studied (Table 2). The high level of genetic diversity suggests that gene pool of barley of Jordan possesses a high level of allelic richness and polymorphism that could be exploited for the improvement and management of barley germplasm. The high level of genetic diversity detected in the present study is possibly a consequence of the location of Jordan in Fertile Crescent which characterized by high diversity of crop plants and even wild plants (Badr *et al*., 2000; Zohary and Hopf, 2000; von Bothmer *et al*., 2003; Pourkheirandish and Komatsuda, 2007).

As estimated by summary statistics, most of the genetic diversity found in this study resided within population rather than between the populations of the studied accessions (Table 2). Moreover, AMOVA analysis revealed a low but a significant genetic diversity between populations, and there was no association between genetic diversity and geographical locations of populations. This suggests that the gene flow between barley from different areas in Jordan is relatively high. These results are in agreement with other studies which revealed a differentiation between different barley accessions from different countries (Ordon *et al*., 1995; Hamza *et al*., 2004). According to AMOVA, the barley landraces could be divided into two groups that reflect different barley inflorescence types, namely six-row in one group and two-row in the second group (Table 3). Based on these results, the six-rowed barley populations significantly differed from two-rowed barley likely as a consequence of the gene flow and selective pressures during domestication and selective (Hamza *et al.*, 2004; Malysheva-Otto *et al.*, 2006; Yahiaoui *et al.*, 2008).

The present study also investigated the gene diversity of barley with the intention of identifying microsatellites that show evidence of selection during barley domestication. To achieve this, a population genetic approach was utilized by starting with SSR genetic loci and asking whether these loci were targets of selection (Doebley *et al*., 2006). Of the 25 microsatellites loci studied here, 9 microsatellites were found to be under possible selection pressures between the two barley phenotypes (two-row vs six-rowed) (Figure 2). These loci possibly represent genes that were selected by barley cultivators as they perhaps contributed to agronomic performance, palatability, nutritional quality, or other traits of barley. It could be hypothesized that these loci were important in the past improvement of barley, and this may indicate that these loci are possible candidates for introgressive breeding from wild relatives to increase the pool of diversity with which modern breeders can work (Vigouroux *et al*., 2002). However, the effect of plant breeding on genetic diversity is a controversial issue. For example, a decrease in genetic diversity because of conventional plant breeding has been found in barley (Graner *et al*., 1990; Allard 1996; Ellis *et al*., 1997; Russell *et al*. 1997). On the other hand, Struss and Plieske (1998) found the same level of genetic diversity in wild and in domesticated barley. Our study has a caveat as we used seeds collected in the past (see Material and Methods section). This makes us cautious as the genetic diversity found in the present study may be different if compared with plant materials that represent the current time. Thus, a further study is needed to compare our results with new barley landraces that represent the current time.

In conclusion, the present study reveals that barley landraces, from different areas of Jordan, posses high levels of genetic diversity and allelic richness that could be utilized in barley improvement and breeding program.

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Phlomis brachydon Essential Oil Against Bacterial Biofilm

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Abstract

Bacteria in biofilms show high resistance to antimicrobial and they cause many persistent and chronic bacterial infections. The failure of antibiotics in eradicating biofilm drives the need for novel approaches to effectively kill bacterial biofilms. Plant essential oils have been used for hundreds of years in traditional medicine to treat infections due to bacteria, fungi, and virus. The aim of the present study is to determine the effects of essential oil of *Phlomis* grown in north Jordan on biofilm-forming bacteria. Six bacterial clinical isolates were used in this study. The Minimum Inhibitory Concentration (MIC) and Biofilm Inhibitory Concentration (BIC) assays were performed in microtitre plates using a twofold dilution series. *Phlomis* essential oil MIC for planktonic bacteria ranged between 0.125 and 2 mg/mL. The most susceptible strains were MRSA and *S. epidermidis.* For bacteria grown in biofilm, the BIC ranged between 0.25 and 4 mg/mL. The most sensitive to *Phlomis* essential oil was *S. epidermidis* while the most resistant were *P. aeruginosa* and *E. coli*. *Phlomis* essential oil was able to inhibit initial adherence in the most tolerant isolate (*E. coli*) at sub-inhibitory concentrations. *Phlomis* essential oil showed a significant activity against all isolates in both planktonic and biofilm growth. It was able to inhibit initial adherence in the most tolerant isolate at sub-inhibitory concentrations.

Keywords: MIC, BIC, biofilm, resistant bacteria, essential oil, Pholims.

1. Introduction

Biofilm is a community of *microorganisms* adhered to surfaces and embedded in a self produced slimy, glue-like matrix. The matrix, which is made up of polysaccharide, protein and DNA, acts as a shield that prevents the access of antimicrobials to biofilm microbes (Veerachamy *et al*., 2014; Stewart and Costerton, 2001). Bacteria in biofilms display a coordinated activity and are able to communicate with each other and exchange signals. Through signalling and sensing, bacteria in a biofilm regulate cell density, formation of channels and pillar like structure for nutrient delivery (Sauer *et al*., 2002; Miller and Bassler, 2001). This is why bacteria in a biofilm are different from planktonic bacteria and they are also found to be different in gene expression (Mikkelsen *et al*., 2007).

Bacterial biofilms show high tolerance to antibiotics and disinfectant hemicals and resist body's defence system. The mechanisms associated with biofilm resistance include: restricted diffusion of antimicrobial agents due to the extracellular matrix, formation of persister cells, adaptive stress responses, slow-growing bacteria (due to depletion of nutrient and oxygen within biofilm) which is less susceptible to antimicrobial agents and expression of biofilm-specific antimicrobial resistance genes (Høiby *et al*., 2010; Patel, 2005).

Biofilm associated *diseases* include cystic fibrosis pneumonia, native valve endocarditis, otitis, bacterial prostatitis, dental plaques, osteomyelitis, musculoskeletal infections, periodontitis and medically associated such as intravascular catheters, urinary catheters and prosthetic implants (Pamp *et al*., 2009; Aparna and Yadav, 2008).

The failure of antibiotics to treat biofilm associated infections increases the need for alternatives. Essential Oils (EO) have been used for centuries in traditional medicine for treating various diseases. Essential oils are very complex mixtures of volatile components produced by aromatic plants as secondary metabolites. They were shown to possess antibacterial, antifungal, antiviral, insecticidal and antioxidant properties (Nazzaro *et al*., 2013; Burt, 2004).

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Predictions about the mode of action of crude essential oils require thorough investigations of their constituents' target site, their mode of action, and their interactions with the surrounding environment (Burt, 2004). As crude essential oils contain many constituents, it is very difficult to predict their mode of action. The strongest antibacterial properties against pathogens come from phenolic compounds such as carvacrol, eugenol and thymol. Essential oils are hydrophobic in nature, which enables them to disturb the bacterial cytoplasmic membrane and mitochondria causing leakage of ions and other cell contents (Hyldgaard *et al*., 2012).

Phlomis is a plant used in herbal medicine for respiratory tract diseases, local treatment of wounds, treatment of ulcers and hemorrhoids. In Jordan traditional medicine *Phlomis brachydon* (Boiss.) Zohary is used to treat stomach and intestine pain (Darwish and Aburjai, 2010). In addition, *Phlomis* have shown activities as anti-inflammatory, immuno-suppressive, antimutagenic, anti-nociceptive, antifibriel, free radical scavenging, anti-malarial, and anti-microbial responses (Sarkhail *et al*., 2006).

There are a few reports about the essential oil constituents of *Phlomis brachydon* (Boiss.) Zohary. To the best of our knowledge, the activity of *Phlomis brachydon* against bacterial biofilm has never been reported worldwide.

The aim of the present study is to determine the effects of essential oil of wild *Phlomis brachydon* (Boiss.) Zohary grown in Jordan on clinical isolates of biofilm-forming bacteria. .

2. Materials and Methods

2.1. Essential oil of Phlomis brachydon

Fresh *Phlomis brachydon* was collected from mountains of Qumeim, Irbid, north Jordan, before the flowering period. The plant materials were taxonomically identified and authenticated by the Botanical Survey of Yarmouk University.

The composition of the essential oil from *Phlomis brachydon* was determined using Gas Chromatography-Mass Spectrometry (GC-MS) (Al-Shuneigat *et al*., 2015). Fifty-eight components accounting for 98.8% of the oil were identified, with oxygenated monoterpenes accounting for about 75% of the total oil content. Major identified compounds were *cis*-chrysanthenol (13.83%), 1,8-cineole (12.84%), *cis*-limonene (12.57%), α-terpinenol (6.97%), and γ-muurolene (4.50%). The concentration of the oil was 0.0032 (wt/wt) and its density was 0.912 g/mL at room temperature (Al-Shuneigat *et al*., 2015).

2.2. Cultures and Media

The effect of *Phlomis brachydon* essential oil on bacterial biofilm was examined using six clinical isolates including: Gram positive bacteria: Methicillin-resistant *Staphylococcus aureus* (MRSA), *Staphylococcus epidermidis*, and *Bacillus subtilis*, and Gram negative bacteria: *Escherichia coli*, *Enterobacter aerogenes*, and *Pseudomonas aeruginosa*. These clinical isolates were isolated from human patients. Cultures were stored on tryptone soya agar (TSA) (Oxoid, Hampshire, UK) at 2-4^0 C and subcultured every 2 months or whenever required. Isolates were purified on specific nutrient agar plates and characterized by standard microbiological and biochemical methods like Gram stain, catalase test, coagulase test and an API system (bioMerieux, France).

2.3. Screening for Biofilm Formation

Biofilm formation was quantified in microtitre plates using the method described by Rachid et al (Rachid *et al*., 2000). Bacteria were grown overnight to mid-log phase by inoculating 10 mL tryptone soya broth (TSB) and incubating at 37ºC until the OD at 600 nm (OD_{600}) reached approximately 0.6. Strains were then diluted in fresh TSB supplemented with 0.5% glucose to give cell density of approximately 10^6 cfu/mL. For each test strain, 200 µL of inoculum was added to 72 wells of a 96-well plate. A quantity of 200 µL TSB was added to the remaining 24 wells and the plate incubated for 24 h at 37^0 C. Following this the optical density at 600 nm (OD_{600}) was measured as an indication of bacterial growth, the plate contents emptied out and washed three times with phosphate-buffered saline (PBS) (Sigma Aldrich). The plates were air-dried and the cells that remained adhered to microwells stained with 0.4% crystal violet (Sigma Aldrich). Optical density at 490 nm (OD_{490}) nm was measured to quantify the amount of crystal violet-stained biofilm. Each strain was assayed in triplicate (Al-Shuneigat *et al*., 2005).

2.4. MIC Assay

MIC was determined using 96 well microtitre plates as described by Rachid *et al*. (2000). Serial two fold dilutions of *Phlomis brachydon* essential oil in TSB were carried out in microtitre plates, 100 µL of bacterial cells with density of approximately 10^6 cfu/mL were added to the wells, mixed and then incubated at 37^{0}C for 24 h aerobically. The MIC was taken as minimal concentration of *Phlomis brachydon* essential oil that inhibited visible growth of the strain. Determination of MIC was carried out in triplicate using three independent experiments. The positive control used for MRSA, *Staphylococcus epidermidis*, and *Bacillus subtilis* was vancomycin, chloramphenicol was used for *E. coli* and *Enterobacter aerogenes* and ceftazidime for *P. aeruginosa*. The absorbance was measured at 600 nm as an indication of bacterial growth.

2.5. Biofilm Inhibitory Concentration (BIC) Assay

BIC was determined using 96 well microtitre plates as described by Rachid et al (Rachid *et al*., 2000). Serial two fold dilutions of *Phlomis brachydon* essential oil in TSB were carried out in microtitre plates, 100 µL of the diluted bacterial cells were added to the wells, mixed and then incubated at 37^{0}C for 24 h aerobically. The wells were washed three times with PBS. The plates were dried using air, and the remaining surface-adsorbed cells of the individual well were stained with 0.1% (w/v) crystal violet. The wells were then washed three times with PBS and allowed to air-dry for 60 min. The crystal violet-stained biofilm was solubilized using 95% (v/v) ethanol and the absorbance read at 490 nm. A well, with no cells and sterile TSB was used as blank (negative control), and

a well with cells and TSB but without *Phlomis brachydon* essential oil was used as a control. The positive control used for MRSA, MSSA, and *S. epidermidis* was vancomycin, for *E. coli* and *K. pneumonia* was chloramphenicol for *P. aeruginosa* was ceftazidime and finally for *P. mirabilis* was ampicillin. BIC was determined as the minimum concentration that caused 30% decrease in optical density. Assays were performed three times on different days for each individual strain and the same result was obtained for each occasion.

2.6. Adherence of Bacterial Cells to Polystyrene

Initial adherence of bacterial cells to polystyrene was determined using a previously reported method (Heilmann *et al*., 1996). Briefly, bacteria were grown overnight in 10 ml TSB at 37^0 C and then diluted 1 : 100 in fresh TSB containing *Phlomis brachydon* essential oil at the required concentration. A quantity of 5 mL of the bacterial suspensions was then poured into Petri dishes and incubated for 30 min at 37^0 C. The plates were washed five times using 5 ml PBS, air dried and stained for 1 min with 0.4% crystal violet. The number of the adhered cells was determined microscopically (CETI 60243T UK) by counting the number of bacteria in 20 fields of view. The essential oil concentrations tested were 1/10 of MIC, 1/2 MIC, and the MIC concentration. Adherence was calculated as the total number of cells adhered per square centimetre examined. Each *Phlomis brachydon* essential oil concentration was assayed in triplicate and the adherence of *Phlomis brachydon* essential oil treated cells compared with untreated controls. Assays were performed three times on different days and the same result was obtained for each occasion

3. Results

3.1. MIC and BIC

Table 1 shows the MIC and BIC values of *Phlomis brachydon* essential oil (mg/mL) for the bacterial isolates used in this study. In general and as expected, the planktonic growth of nearly all bacteria strains tested were more sensitive to *Phlomis brachydon* essential oil than biofilm growth.

The MIC values for planktonic growth were between 0.125 and 2 mg/mL. The most susceptible strains were MRSA and *S. epidermidis.*

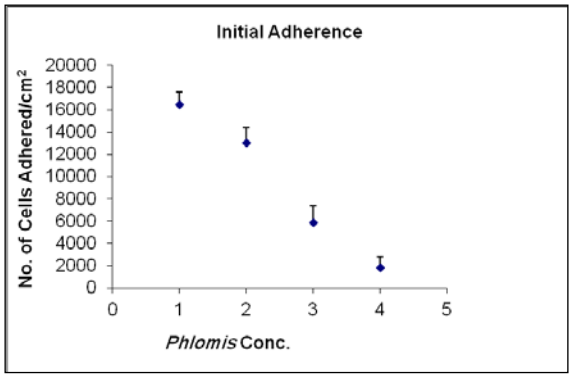
For biofilm, the BIC values were between 0.25 and 4 mg/mL. The most sensitive to *Phlomis* essential oil was *S. epidermidis* while the most resistant were *P. aeruginosa* and *E. coli*. planktonic *E. coli* was very sensitive to *Phlomis* essential oil and the most resistant to *Phlomis* essential oil grown as biofilm

Table 1. MIC and BIC of *Phlomis* (mg/mL) for the bacterial isolates used for this study.

Isolate Number	Isolate name	MIC_{plank}	$BIC_{biofilm}$
1	MRSA	0.125	2
2	*P. aeruginosa*	1	4
3	*E. aerogenes*	2	2
4	*E. coli*	0.25	4
5	*B. subtilis*	2	2
6	*S. epidermidis*	0.125	0.25

3.2. Inhibition of E. coli Adherence to Polystyrene by Phlomis at sub-MIC Levels

As stated above, *E. coli* was very sensitive to *Phlomis brachydon* essential oil in planktonic growth and became very resistant in biofilm growth. Because of the great variations between MIC and BIC values and because BIC value for *E. coli* was one of the highest, *E. coli* was chosen to test the effect of sub-inhibitory concentrations (sub-MIC_{plank}) on its initial adherence to polystyrene.



The results show that adding sub-inhibitory concentrations (sub-MIC_{plank}) of *Phlomis* essential oil to polystyrene Petri dishes containing a suspension culture of the *E. coli* strain were able to reduce the number of individual cells adhering to the polystyrene surface after 30 minutes incubation period (Fig. 1).

Figure 1. Effect of *Phlomis* on initial adhesion of *E. coli*; 1: without EO, 2: 1/10×MIC 3: 1/2×MIC, 4: MIC

4. Discussion

Biofilm is a complex structure that causes chronic infections. Biofilm infections are extremely resistant to antibiotics and conventional antimicrobial agents, and are able to evade host defenses. Nowadays, the known antibiotics became of limited effectiveness in treating biofilms infection. Biofilms are able to withstand 100 to 1000 times the concentrations of antibiotics that can inhibit planktonic cells (Wolcott and Ehrlich, 2008). This means that there is an urgent need for the development of alternatives to antibiotics. A possible alternative is plants derived essential oils. Essential oils have been shown to possess broad-range of antibacterial properties (Hyldgaard *et al*., 2012; Oussalah *et al*., 2007).

Essential oils have been used in folk medicine since ancient times for the treatment of various diseases. The

main cellular targets of essential oil are cell membrane and cytoplasm (Nazzaro *et al*., 2013).

The aim of the present study is to test the effect of essential oil of wild *Phlomis brachydon* on biofilm-forming bacterial clinical isolates. The results show that the MIC values for planktonic growth were between 0.125 and 2 mg/mL while the BIC values were higher for nearly all strains.

The essential oil of *Phlomis brachydon* has been able to overcome the resistance mechanisms of the plankonic growth of two of the most prominent antibiotics resistant pathogens MRSA with MIC of only 0.125 mg/mL and *P. aeruginosa* with MIC of 1 mg/mL.

The results show that in general that the MIC_{plank} values are lower than that of $BIC_{biofilm}$. This is not unexpected as pathogens become more resistant in biofilm than in planktonic form. The essential oil of *Phlomis* was able to overcome the resistance of *S. epidermidis* at very low concentrations in both planktonic and biofilm form. *S. epidermidis* is a very important opportunistic pathogen and the most common source of infections on indwelling medical devices (Otto, 2009). Each year, billions of dollars are spent to replace the infected devices such as intravascular catheters, mechanical heart valves, pacemakers, prosthetic joints, contact lenses, urinary catheters and prosthetic implants (Chen *et al*., 2013).

Sub-inhibitory concentrations (sub-MIC_{plank}) of *Phlomis brachydon* essential oil to polystyrene Petri dishes containing a suspension culture of the *E. coli* strain were able to reduce the number of individual cells adhering to the polystyrene surface. In general, bacterial biofilm formation occurs in two stages (Chen *et al*., 2013; Mack, 1999). The initial phase coincides with adhesion of bacteria to the biomaterial surface and this phase is reversible. It is then followed by an irreversible second phase of cell to cell accumulation as multilayered cell clusters and biofilm form. The reduced bacterial adherence to polystyrene caused by PT can be explained by either the alteration of adherence factors present on the bacterial cell surface or by the modification of the polystyrene surface. Such effects could be unrelated to the mechanism by which the essential oil inhibits growth and may only be produced by relatively high levels of the essential oil.

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A Statistical Design Approach for Xylanase Production by *Aspergillus niger* Using Soybean Hulls: Optimization and Determining the Synergistic Effects of Medium Components on the Enzyme Production

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Abstract

Xylanases are hydrolytic enzymes produced by different microorganisms which convert xylooligomers to its constituent xylose units. The growing interests in xylanase production could be linked to its diverse industrial applications. The use of agricultural residues contributed in overcoming one of the major challenges of enzyme production, which is the production cost. This study involves the use of Face centered central composite design (FCCCD) under Response surface methodology (RSM) to optimize the medium components for enhanced xylanase production using soybean hulls as renewable substrate by solid state fermentation. The optimum components that led to maximum xylanase activity of 139.73 U/g were 0.58% w/w of urea, 0.03% w/w of K_2HPO_4 and 0.04% w/w of Na_2CO_3. Coefficient of determination (R^2) used to check the fitness of the model showed the correlation between the experimental and predicted responses with a value of 0.8981. Thus, this study showed the potential use of soybean hulls for xylanase production

Keywords optimization, renewable substrate, soybean hulls, xylanase.

1. Introduction

Xylanase (endo-1, 4-β-D-xylanohydrolase; EC 3.2.1.8) is a xylan-degrading enzyme that aids in hydrolyzing xylan into its monomeric units (Mittal *et al.*, 2013). This enzyme is produced by various microbial systems including bacteria, yeast and fungi (Maheshwari *et al.*, 2014). The growing interest in xylanase production includes its utilization for various industrial applications such as biobleaching of pulp and paper, clarification of wine and juice, animal feed formulation, baking processes, and hydrolysis of lignocellulose for ethanol and xylitol production, among others (Bibi *et al.*, 2014; Coman and Bahrim, 2011).

Xylanase production is affected by various factors including the cost of raw materials, product inhibition, pH and thermal stability, as well as polymeric nature of xylans (Walia*et al.*, 2013). Although different agricultural substrates, such as wheat bran (Coman and Bahrim, 2011), tomato seed meal (Katapodis *et al*., 2006), apple pomace (Walia *et al.*, 2013), wheat straw (Garg *et al.*, 2011), sugarcane bagasse (Souza *et al.*, 1999), rice straw (Park *et al.*, 2002), rice husk (Singh *et al.*, 2013), sunflower meal and corn cobs (Irfan *et al.*, 2014), have been used for the production of xylanase through solid state fermentation as parts of the efforts on cost reduction, there is still a need for developing an effective fermentation system for enhanced levels of this enzyme for wider industrial applications.

Soybean, as a widely cultivated crop across the globe, has an annual production of more than 259 million tonnes; generating more than 20 million tonnes of hulls; thus, soybean hulls consist of 46–51% cellulose, 16–18% hemicellulose and 1.4–2% lignin (Corredor *et al.*, 2008; Cassales *et al.*, 2011); these make them attractive for ~~the~~ production of several value added products. Thus, xylan, being a major component of hemicellulose present in soybean hulls, could serve as an inducer for xylanase production.

Herein, Face Centered Central Composite Design (FCCCD) was employed for enhancing xylanase production using soybean hulls as a cheap and available substrate by *Aspergillus niger* AS-1. Preliminary screening of medium components using Placket-Burman design and one-factor-at-a-time method has been established by Salihu and Alam (2015). This design was selected based on its reliability with the ability to identify the separate and combined effects of various factors through a minimum number of experiments.

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2. Materials and Methods

2.1. Sample and Inoculum preparation

Soybean hulls of 1 mm particle sizes were prepared and kept under laboratory conditions at room temperature. A spore suspension of *Aspergillus niger* AS-1 cultured on potato dextrose agar plate was prepared as described by Alam *et al.* (2004) using 25 ml of sterile distilled water with the help of bent glass rod. Following the filtration of the suspension through Whatman No.1 filter paper under aseptic conditions; the spore count was found to be about 1×10^8 spores/ml and used as an inoculum in this study

2.2. Solid state fermentation and Xylanase production

The fermentation set up for xylanase production was prepared using 10 g of soybean hulls in 250 ml Erlenmeyer flask which was mixed with 10 ml of a mixture of urea, K_2HPO_4 and Na_2CO_3 at a different percentages (w/w) (as shown in Table 1), 0.5% w/w of peptone, 0.05% w/w each of $NaNO_3$, $(NH_4)_2SO_4$ and $CaCl_2$; so as to get the final solid to moisture ratio of 1:1 as described by Xu *et al.* (2008). The flasks were autoclaved at 121^0C for 20 min, and then cooled before inoculating with 1 ml of spore suspension of *A. niger* AS-1 (1×10^8 spores/ml). The set ups were incubated at 28 ± 1^0C for 5 days. Following the bioconversion, distilled water was added to each flask and then shaken on a rotatory shaker (180 rpm) for 30 min at room temperature. The content was centrifuged and the supernatant was used to assay for xylanase activity.

2.3. Determination of Xylanase activity

Xylanase activity was determined using xylan from beech wood as a substrate and the amount of reducing sugar released was measured by dinitrosalicylic acid (DNS) method using D-xylose as the standard (Biely *et al.*, 1985). The reaction mixture contains 900µl of 1% (w/v) xylan in 0.05 M citrate buffer, pH 5 and 100 µl of the enzyme solution incubated at 50^0C for 30 min. The reaction was terminated by the addition of DNS followed by incubating the tubes in boiling water bath for 5 min. The reducing sugar released was measured at 540 nm. One enzyme unit (U) was defined as the amount of enzyme that liberated 1 µmol of xylose per minute under the assay conditions. The results were expressed as U/g soybean hulls.

2.4. Statistical analysis and optimization

Face Centered Central Composite Design (FCCCD) under Response Surface Methodology (RSM) was used (Montgomery, 2001) to determine the effects of the medium components influencing xylanase production. Three medium components (urea, K_2HPO_4 and Na_2CO_3) were selected for the present study based on the results of Placket-Burman design and One-factor-at-a-time (OFAT) analysis (Salihu and Alam, 2015). Design Expert Statistical software (Version 6.0.8) was used to generate a set of seventeen (17) experiments with three center points so as to optimize the concentrations of these parameters on xylanase activity. The parameters were studied at three levels, low (–1), medium (0) and high (+1) as indicated in Table 1. The relationship between the dependent and independent variables is based on second order polynomial equation:

$$Y = \beta_o + \beta_1X_1 + \beta_2X_2 + \beta_3X_3 + \beta_{12}X_1X_2 + \beta_{13}X_1X_3 + \beta_{23}X_2X_3 + \beta_{11}X_1^2 + \beta_{22}X_2^2 + \beta_{33}X_3^2 \quad (1)$$

where Y represents the dependent variable (xylanase production); X_1, X_2 and X_3 are independent variables (urea, K_2HPO_4 and Na_2CO_3) ; β_0 is an intercept term; β_1, β_2 and β_3 are linear coefficients; β_{12},β_{13} and β_{23} are the interaction coefficients; β_{11},β_{22} and β_{33} are quadratic coefficients.

The results obtained were used to develop a regression model by analyzing the values of regression coefficient and analysis of variance (ANOVA). The quality of fit of the quadratic polynomial model equation was expressed by coefficient of determination, R^2. The validation experiment was carried out to determine the adequacy of the developed experimental model.

3. Results and Discussion

The present study involves the use of statistical design to determine the relationship between the selected medium components (urea, K_2HPO_4 and Na_2CO_3) for enhanced xylanase production. Through this design, linear and interactive effects of these components on the response could be ascertained.

Table 1 shows the FCCCD design with both the experimental and predicted response for the 17 experimental runs which constitute 8 star points, 6 axial points and 3 center points. The results showed that highest activity of xylanase (137.58 -140.17 U/g) was realized in runs associated with center points (6, 8 and 15), while the lowest was found in run 17 (113.61 U/g), where all the three components were at their low levels.

The relationship between the medium components and the response was predicted by the second-order polynomial equation in terms of coded factors:

$$\text{Xylanase activity (U/g)} = +139.03 - 0.16A + 0.26B + 3.49C - 5.24A^2 - 2.66B^2 - 5.89C^2 - 2.71AB - 1.49AC - 0.27BC \quad (2)$$

where Xylanase production is dependent on concentrations of urea (A), K_2HPO_4 (B) and Na_2CO_3 (C). The negative and positive signs before the terms show antagonistic and synergistic effects, respectively. The analysis of variance (ANOVA), as indicated in Table 2, helps in assessing the fitness as well as the significance of the developed model. As indicated in the Table, the model was found to be significant with *p*-value of 0.0095; while the linear term of Na_2CO_3 (C), quadratic terms of urea (A^2) and Na_2CO_3 (C^2) were significant at *p*-value less than or equal to 0.05.

The lack of fit was not found to be significant based on its *p*-value of 0.1008, as shown in Table 2; this indicates that there is only a 10.08% chance that this value could occur due to noise.

Similarly, coefficient of determination (R^2) is used to check the fitness of the model, the closer a value is to 1, the better the correlation between the experimental and predicted responses. Thus, R^2 value of 0.8981 and adjusted R^2 value of 0.7670 indicate that about 90% and 77% of variations could be accounted for by the model equations. Adequate precision value of 8.22 which measures the signal to noise ratio showed an adequate

signal since any value greater than 4 is considered desirable. In case of coefficient of variation, lower values are preferred as they indicate the precision and reliability of the experimental responses as presented in foot notes of Table 2.

Figure 1 shows the two and three dimensional surface plots of predicted xylanase production based on urea and K_2HPO_4. Although none of the interaction terms was significant, as indicated in Table 2, the AB (urea and K_2HPO_4) was the one with the lowest *p*-value (0.0627). It can be seen from ~~the~~ Figure 1, that xylanase production is affected by ~~the~~ increasing concentration of urea and K_2HPO_4, but at higher concentrations, xylanase production was found to be low.

In order to investigate the applicability of the developed second-order model based on the three parameters (urea, K_2HPO_4 and Na_2CO_3), numerical optimization was used to determine the most desirable concentrations of the components that lead to maximum outcome for validation (Table 3a). The desirable model solution was validated as indicated in Table 3b; the experimental and the predicted results appeared to be in good agreement.

Accordingly, the concentrations of the three medium components optimized in the present study were lower than those reported by Xu *et al.* (2008) for maximum xylanase production using wheat bran and *A. niger* XY-1 at optimum concentrations of urea, Na_2CO_3 and $MgSO_4$ of 4.2%, 0.3% and 1.1%, respectively.

Park *et al.* (2002) reported a combined optimization based on medium and process parameters by fractional factorial design that led to enhanced xylanase activity of 5,071 U/g using rice straw by *A. niger*. The yield was several times higher than what was observed in the present study, but there is a possibility of getting an enhanced production in soybean hulls containing medium when considering some parameters such as temperature, moisture content, inoculum concentration, pH, pretreatment methods and particle size. Similarly, xylanase yield of 10.9 U/ml was obtained following CCD analysis of alkali-pretreated rice husk by *A. niger* ITCC 7678. The optimum operating conditions were found to be pH of 5.5, temperature of 32.5 °C, and $NaNO_3$ of 0.35 % (w/v) (Singh *et al.*, 2013).

In case of sugarcane bagasse, effects of process parameters were analyzed using RSM for enhanced xylanase production by *Thermoascus aurantiacus*. Initial moisture content of 81% and bagasse mass of 17g influenced xylanase activity with a maximum yield of 2700 U/g (de O Souza *et al.*, 1999). Based on these findings, de O Souza *et al.* (1999) suggested that xylanase production by several fungal species using agricultural residues depends on the composition of the substrates, choice of fermentation type and conditions as well as downstream processing of the produced enzyme.

Ang *et al.* (2013) reported the potential of untreated oil palm trunk for xylanase production by *A. fumigates* SK1. High extracellular xylanase activity of 418.70 U/g was obtained when the moisture content, initial pH and inoculum concentration were at 80%, 5.0 and 1×10^8 spore/g, respectively. When a 10 liter-capacity stainless steel horizontal bioreactor was used for the production of xylanase by *Sporotrichum thermophile* in an optimized medium containing wheat bran and wheat straw; maximum xylanase yield was found to be 320 U/g (Topakas *et al.*, 2003). Additionally, application of CCD resulted in an enhanced xylanase production by *Cellulosimicrobium* sp. CKMX1 using apple pomace based medium. Some of the parameters that supported xylanase production include particle size, fermentation time, temperature, initial pH and inoculum concentration; with a maximum yield of 535.6 U/g (Walia *et al*., 2013).

Thus, the findings of the present study indicate that FCCCD was suitable for optimizing the medium components for enhanced xylanase production by *A. niger* AS-1 using soybean hulls; further studies involving the process parameters will give the overall picture in terms of yield and production cost economics of the process.

Table 1: Face centered central composite design matrix indicating the experimental and predicted responses

Run	Urea (% w/w)	K_2HPO_4 (% w/w)	Na_2CO_3 (% w/w)	Xylanase activity (U/g)	
				Experimental	Predicted
1	0.8 (+1)	0.04 (+1)	0.03 (–1)	117.74	120.92
2	0.8 (+1)	0.03 (0)	0.04 (0)	132.74	133.63
3	0.6 (0)	0.03 (0)	0.05 (+1)	133.51	136.63
4	0.8 (+1)	0.02 (–1)	0.05 (+1)	128.23	129.81
5	0.8 (+1)	0.02 (–1)	0.03 (–1)	127.33	125.26
6	0.6 (0)	0.03 (0)	0.04 (0)	139.33	139.03
7	0.8 (+1)	0.04 (+1)	0.05 (+1)	127.95	124.37
8	0.6 (0)	0.03 (0)	0.04 (0)	140.17	139.03
9	0.6 (0)	0.02 (–1)	0.04 (0)	136.03	136.11
10	0.4 (–1)	0.03 (0)	0.04 (0)	134.84	133.95
11	0.6 (0)	0.03 (0)	0.03 (–1)	132.77	129.65
12	0.4 (–1)	0.04 (+1)	0.05 (+1)	131.01	133.08
13	0.6 (0)	0.04 (+1)	0.04 (0)	136.71	136.63
14	0.4 (–1)	0.02 (–1)	0.05 (+1)	130.87	127.69
15	0.6 (0)	0.03 (0)	0.04 (0)	137.58	139.03
16	0.4 (–1)	0.04 (+1)	0.03 (–1)	125.26	123.68
17	0.4 (–1)	0.02 (–1)	0.03 (–1)	113.61	117.19

Table 2: Analysis of variance (ANOVA) of the developed model

Source	Sum of squares	F-value	*p*-value	Remark
Model*	739.4869	6.8516	0.0095	Significant
A (Urea)	0.2562	0.0214	0.8879	Not significant
B (K_2HPO_4)	0.6762	0.0564	0.8191	Not significant
C (Na_2CO_3)	121.5252	10.1338	0.0154	Significant
A^2	73.4355	6.1237	0.0425	Significant
B^2	18.8904	1.5752	0.2497	Not significant
C^2	92.8006	7.7385	0.0272	Significant
AB	58.6469	4.8905	0.0627	Not significant
AC	17.7026	1.4762	0.2638	Not significant
BC	0.6047	0.0504	0.8287	Not significant
Lack of Fit	80.4522	9.2154	0.1008	Not significant

*R^2 = 0.8981, Adjusted R^2 = 0.7670, Coefficient of variation (CV) = 2.65, adequate precision = 8.22

Table 3a: Constrains for the parameters in numerical optimization

Parameters	Ultimate goal	Experimental range
Urea	range	0.4 – 0.8 %
K_2HPO_4	range	0.02 – 0.04 %
Na_2CO_3	range	0.03 – 0.05 %
Xylanase production (U/g)	Maximum production	113.61 – 140.17 U/g

Table 3b: Optimization and model validation

Urea (% w/w)	K_2HPO_4 (% w/w)	Na_2CO_3 (% w/w)	Xylanase production (U/g)	
			Experimental	Predicted
0.58	0.03	0.04	139.73 ± 0.19	139.572

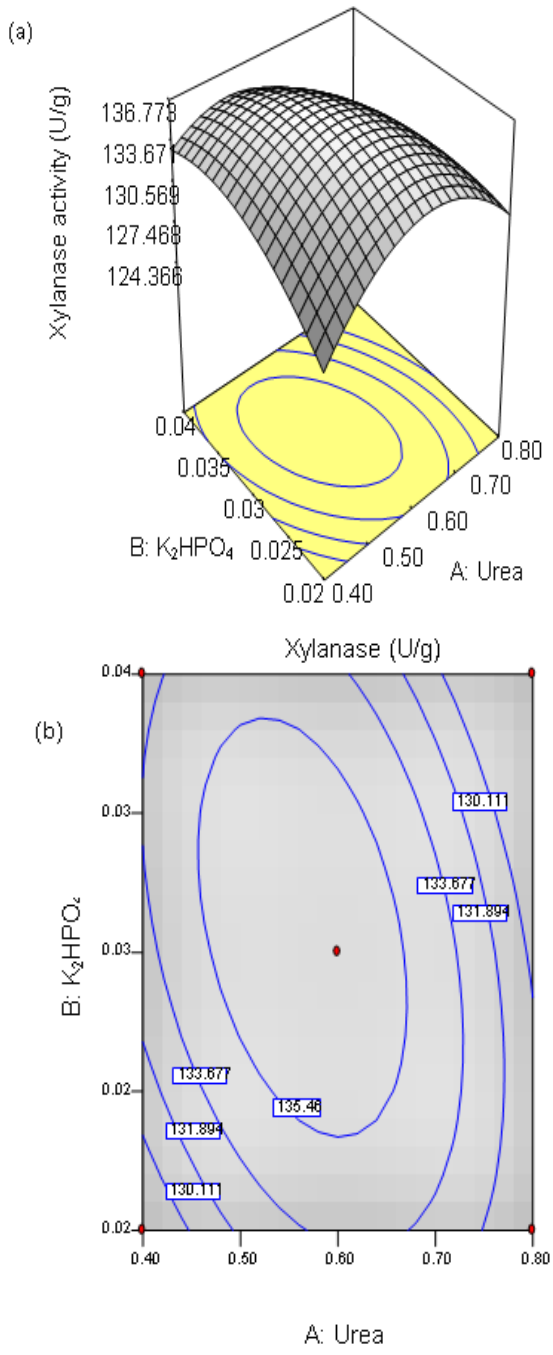

Figure 1: Plots of the effects of urea and K_2HPO_4 on xylanase activity by *A. niger* AS-1 using soybean hulls: (a) response surface 3D and (b) contour plot (2D).

4. Conclusion

Statistical optimization using FCCCD for xylanase production by *A. niger* AS-1 using soybean hulls as raw material indicated the dependence of the production on the selected medium components (urea, Na_2CO_3 and K_2HPO_4). Under the optimized condition, maximum xylanase activity of 139.73 U/g was obtained. Thus, the utilization of soybean hulls as an inexpensive and renewable substrate for xylanase production boosts its industrial relevance when compared with its current usage.

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A Note on the Karyotype of the Amphibian *Pelophylax bedriagae* from Jordan

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Abstract

New data are presented on the karyotype of the Levant Water Frog, *Pelophylax bedriagae*, from Jordan. 26 chromosomes were observed for both male and female *Pelophylax bedriagae*. Seven chromosomes are metacentric (chromosomes 1, 3, 5, 6, 7, 8 and 9) and six are submetacentric (chromosomes 2, 4, 10, 11, 12 and 13). This is the first study on the karyotype of the Jordanian amphibians.

Keywords Karyotype, amphibians, *Pelophylax bedriagae*, Jordan

The diversity of the amphibians of Jordan is relatively low, consisting of three extant species (Disi & Amr, 2008). The taxonomic status of amphibians has undergone radical changes over the past 10 years. Recent molecular investigations on the amphibians of the Palaearctic region yielded new insights to the taxonomic status of the amphibians of the Middle East and Jordan in particular. For example, Plötner *et al*. (2001) showed that the Levant Water Frog, *R. bedriagae*, in Jordan and Syria is distinct from the European Water Frog, *Rana ridibunda*, and the Anatolian *R. bedriagae* differs from the southern populations by 2.2-3.4% of the analysed mitochondrial DNA. Lymberakis *et al*. (2007) compared partial mitochondrial DNA sequences for the cytochrome b and 16S rRNA genes for *Rana* populations in the Eastern Mediterranean region. They consider that within the *rindibunda*/*bedriagae* lineage, *R.* (*P.*) *ridibunda*, *R.* (*P.*) *epeirotica*, *R.* (*P.*) *cretensis*, *R.* (*P.*) *bedriagae*, *R.* (*P.*) *cerigensis* and *R.* (*P.*) *kurtmuelleri* were differentiated from a common ancestor through a series of vicariant and dispersal events.

In the Middle East, some studies reported on the karyotype of amphibians, including the former *Rana ridibunda* (=*Pelophylax bedriagae*). Studies were performed in Saudi Arabia (Al-Shehri & Al-Saleh, 2005), Palestine (Salman *et al*., 2015), and Turkey (Alpagut & Falakali, 1995).

The present study is the firsts to report on the karyotype of *Pelophylax bedriagae* collected from Jordan.

Four adult *Pelophylax bedriagae* were collected from a pool at the Jordan University of Science and Technology campus. Frogs were injected with 0.2 ml colchicine solution (1 mg/ml) for 6-12 hours before the bone marrow of the femoral bones was removed for the cytogenetic study. The bone marrow was washed by using 0.9% KCl hypotonic solution into centrifuge tube, then incubate for 15 minutes at 37^0 C. The homogenate was centrifuged for 6-8 minutes at 1000 rpm, and then fixed using fresh fixative (3 volumes of methanol+1 volume of glacial acetic acid). The supernatant was removed by a pipette as much as possible, keeping the bone marrow pellet. More fixative was added and then centrifuged again 6-8 minutes at about 1000 rpm. The same procedure was repeated three times. The pellet was suspended in a small volume of the fixative and then dropped on clean microscopic slide. Slides were stained in giemsa solution in phosphate buffer for 12-15 minutes.

A minimum of five metaphases were analyzed for each specimen and images were taken using an Olympus MX41 microscope fitted with a digital camera. Standard karyotypes were constructed from micrographs of well spread chromosomes. Metaphase chromosomes were arranged in homologous pairs according to size and centromere position. The diploid chromosome number was determined from ten photographed spreads for each species.

Twenty-six chromosomes were observed for both male and female *Pelophylax bedriagae* (Fig. 1). The karyotype of both sexes are similar and cannot be differentiated from each others. Seven chromosomes are metacentric (chromosomes 1, 3, 5, 6, 7, 8 and 9) and six are submetacentric (chromosomes 2, 4, 10, 11, 12 and 13).

Species of the genus *Rana* in both the old and new worlds have 2n=26 (Haertel *et al*., 1974; Nishioka *et al*., 1987). Our results are similar to those obtained by Al-Shehri & Al-Saleh (2005) where they reported seven metacentric and six submetacentric chromosomes, and to those reported by Salman *et al*. (2015). We were unable to differentiate the sex chromosomes.

Alpagut & Falakali (1995) showed that the karyotypes of two Turkish populations of *Rana ridibunda* consisted of 26 chromosomes (2n=26), with a similar chromosomal

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morphology. However, they indicated that there were heteromorphic male sex chromosomes.

Further studies should investigate the taxonomic status of the three amphibian species known in Jordan, both for the molecular structure and morphology of their chromosomes, including G-banding.

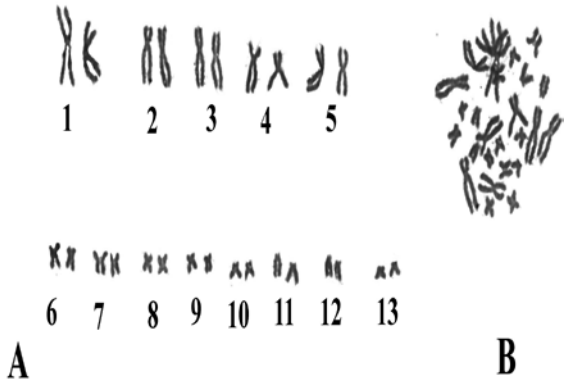

Figure 1: **A.** Karyotype for male *Pelophylax bedriagae.* **B.** Metaphase plate.

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