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EDITORIAL PREFACE

Jordan Journal of Biological Sciences (JJBS) has had another great year. We have seen a significant increase in articles submission from both regional and international scholars. The editorial board members of JJBS have been very busy throughout the year to maintain excellence in the quality publication of accepted papers. As a result, JJBS has been indexed by CABI's Full-Text Repository, EBSCO and is currently under evaluation to be indexed in National Library of Medicine's MEDLINE\ PubMed system and Elsevier's SciVerse Scopus.

As in the previous two years, this sixth volume of JJBS will include four issues, ten to twelve articles in each issue. In the coming year, it is my vision to have JJBS publishes more outstanding papers and review articles from distinguished scholars in various areas of biological sciences. In addition, I will be working on the inclusion of JJBS in ISI, which will lead to a wider readership and good impact factor. As you read throughout this inaugural volume of JJBS, I would like to remind you that the success of our journal depends directly on the number of quality articles submitted for review. Accordingly, I would like to request your participation by submitting quality manuscripts for review and by encouraging your colleagues to do the same. One of the great benefits we can provide to our prospective authors, regardless of acceptance of their manuscript or not, is the mentoring nature of our review process. JJBS provides authors with high quality, helpful reviews that are shaped to assist authors in improving their manuscripts.

I would like to thank the JJBS International Advisory Board members for their continuous support of JJBS. Furthermore, I would like to thank the JJBS Editorial Board members for their exceptional work and continuous support to JJBS. My thanks are also extended to the Hashemite University and Jordanian Scientific Research Support Fund for their continuous financial and administrative support to JJBS.

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

Prof. Khaled H. Abu-Elteen
Editor-in-Chief
Hashemite University
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Fermentation Studies for the Production of Dibutyl Phthalate, an Ester Bioactive Compound from *Streptomyces albidoflavus* MTCC 3662 Using Low-Priced Substrates

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Abstract

Attempts were made to evaluate the limiting nutritional parameters for production of dibutyl phthalate from *Streptomyces albidoflavus* MTCC 3662. The compound was found as a broad spectrum bioactive compound that acts as an antimetabolite of proline. Among the carbon and nitrogen sources tested in batch fermentation, glycerol (1.75%) and arginine (0.075%) supported best production. Arginine was replaced with sodium nitrate (0.025%) in the later studied due to its low cost, easy availability and as well as for good production capability. Other limiting nutrients, KH_2PO_4 0.05%, NaCl 0.15% and $MgSO_4$ 0.03% were found best for production. Effect of trace salts in the basal medium was also tested. Medium supplementation with casein hydrolysate, jaggery and yeast extract (0.001%, each) enhanced the production. Yield enhancement was also observed with Tween-80 (0.2µg/ml). Optimization of nutritional parameters imparts enhancement of production by 2.4 fold, using low cost substrates.

Keywords: Fermentation, Optimization, Dibutyl Phthalate, Bioactive Compound, Antimetabolite, Proline, *Streptomyces albidoflavus* MTCC 3662

1. Introduction

Dibutyl phthalate (DBP) is bioactive ester produced by bacteria (Morsi *et al*., 2010), actinomycetes (Roy *et al*., 2006), fungi (Mabrouk *et al*., 2008), algae (Namikoshi *et al*., 2006; Babu and Wu, 2010) and also by higher plants (Ruikar *et al.,* 2011). DBP varies qualitatively in terms of ^{14}C content of various sources between petrochemicals and biologicals. In case of biological source, ^{14}C level is more than 50% whereas of petrochemical source, it is beyond the detectable level (Namikoshi *et al.,* 2006). The *de novo* synthesis of DBP by plant cell culture using medium containing $NaH^{13}CO_3$ has been reported (Babu and Wu, 2010). The bioactive compound produced by *Streptomyces albidoflavus* MTCC 3662 was structurally similar to DBP having antimicrobial activity against Gram positive and Gram negative bacteria, and also against unicellular and filamentous fungi (Roy *et al.,* 2006). It is also a pH and thermo tolerant antimetabolite of proline (Roy *et al*., 2006, Roy and Sen, 2007). Antimetabolites, the potential antimicrobial, are organic compounds having deleterious effect on g rowth and viability of an organism while the effect can be reverted by concurrent administration of one or more common biochemicals (Pruess and Scannell, 1974). They act as anticancer and antitumour (Ogawa *et al*., 1998) and herbicidal (Lee *et al*., 2003) compounds and also in studies of various physiological processes. Cytotoxic activity of DBP against tumor cell lines has been worked out (Mabrouk *et al.,* 2008). Thus, this necessitates further studies on DBP production.

There is no c ommon phenomenon or regulation connected with the growth and production ability of microorganisms. Under varying conditions of cultivation, the production may be stimulated. Suitable alterations of carbon, nitrogen or phosphate sources and supplements have been reported to affect metabolic processes in streptomycetes (Barratt and Oliver, 1994; Loun'es *et al*., 1996; Abbanat *et al*., 1999). Therefore, it is essential to pay special attention for the optimization of production parameters for cost effective production of DBP. Optimization of physical conditions of the selected medium for the production of DBP by *S. albidoflavus* MTCC 3662 has been reported (Roy and Sen, 2011). This communication evaluates the effect of nutritional factors for production of DBP by *S. albidoflavus* MTCC 3662.

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2. Materials and Methods

2.1. Microorganisms Used

The *Streptomyces albidoflavus* 321.2 (MTCC 3662), a soil isolates (Roy and Sen, 2002), was maintained in glucose-asparagine agar containing (g/l): glucose, 10; asparagine 0.5; K_2HPO_4, 0.5; pH 6.8; agar, 20. Test organism *Escherichia coli* ATCC 25922 was maintained in nutrient agar, containing (g/l) peptone, 5; NaCl, 5; beef extract, 1.5; yeast extract 1.5 pH 7.2; agar, 20. Organisms were stored at 4°C.

2.2. Inoculum

Spore suspension ($1.2x10^7$spores/ml) of 6 da y old culture was used as inoculum.

2.3. Basal Production Medium

Arginine glycerol salt (AGS) medium, containing (g/l) arginine, 1; glycerol, 12.5; K_2HPO_4, 1; NaCl, 1; $MgSO_4$, $7H_2O$ 0.5; $Fe_2(SO_4)_3$, $6H_2O$ 0.01; $CuSO_4$, $5H_2O$ 0.001; $ZnSO_4$, H_2O 0.001; $MnSO_4$, H_2O 0.001; agar 20; pH 6.75. All chemicals used were of analytical grade.

2.4. Fermentation Condition

Batch experiments were carried out in basal medium (25 ml in 100 ml Erlenmeyer flask) at 32°C temperature for 6 da ys at stationary condition using 6% inoculum. Other test chemicals for individual experiments were sterilized separately and added to the fermentation medium in required quantity aseptically. The optimized parameter of an experiment was considered for the designing of subsequent experiments keeping all other conditions constant. All the experiments were made in five set in all treatments and each test case.

2.5. Assay of Antimetabolite

Yield of antimetabolite was determined by agar cup method (Higashida *et al*., 1971), using glucose-asparagine agar and 1ml suspension ($1.2X10^6$ CFU/ml) of the test organism. Each cup was filled with cell-free fermented broth (crude sample, 0.1 ml) and incubated at 30°C for 24 hs. The yield was determined by measuring the zone of inhibition against a standard curve of authentic sample.

2.6. Statistical Analysis

Standard error (±) of mean was calculated from five determinations using Microsoft Office Excel 2007.

3. Results and Discussion

3.1. Effect of Carbon Source

In batch experiments production kinetics were observed under growth-limiting conditions. Carbon being the prime factor for growth, several carbon sources was tried, taking 1% of each along with 0.32 g/l of nitrogen, as recommended in AGS medium. The isolate was able to grow in all the tested carbon sources, however; maximum production was obtained in medium containing glycerol as a sole source of carbon followed by Ca-gluconate and lactose (Table 1). In medium, containing maltose, meso-inositol, Na-acetate and xylose, the production was not detectable. It was further observed that increase of glycerol amount above 1.75% influenced the yield, causing inhibition (Figure 1). Authors worked for secondary metabolite production by *Streptomyces* spp. found glycerol limitation at 1% (Kojima *et al*., 1995), 1.15% (Bhattacharyya *et al*., 1998) and 2% (Poetsch *et al*., 1985).

Table 1. Suitability of carbon source for the production of DBP by *Streptomyces albidoflavus* MTCC 3662

Carbon sources (1%)	Yield (µg/ml)
Na-acetate	ND
D-Ribose	75 ±1.58
Xylose	ND
Raffinose	140±2.74
Ca-gluconate	175±3.54
Dextrose	75±1.58
Meso-inositol	ND
Mannitol	95±1.58
Glucose	110±2.74
Fructose	70±1.58
Mannose	90±2.24
Galactose	125±3.54
Glycerol	250±3.87
Sucrose	110±2.24
Lactose	145±3.16
Maltose	ND
Starch	070±1.58
Control (without carbon source)	ND

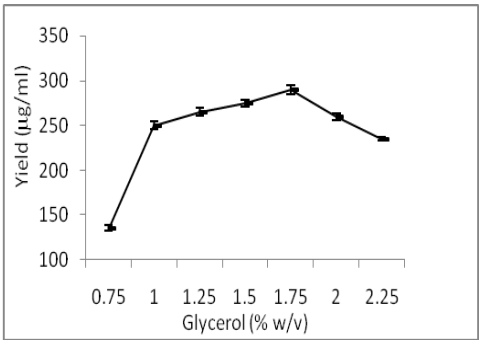

Figure 1. Effect of glycerol concentration on the production of DBP by *Streptomyces albidoflavus* MTCC 3662.

3.2. Effect of Nitrogen Source

Preferential utilization of nitrogen is a distinguishing phenomenon of *Streptomyces.* Nitrogen sources (0.32N g/l) were tested with optimum level of glycerol. It was revealed that the yield was greatly influenced by the nature of the nitrogen source (Table 2). Arginine was the best source for yield enhancement followed by asparagine and sodium nitrate. Subsequent repeat experiment was performed with the best two nitrogen sources. It was found that 0.075% arginine and 0.025% sodium nitrate were the most favored sources for yield (Figure 2). Of the two nitrogen sources, sodium nitrate was selected as a cheap and easy availability. Several researchers (Furumai *et al*., 1993; Haque *et al*., 1995, Bhattacharyya *et al*., 1998) studied the nitrogen limitation and secondary metabolite production by *Streptomyces* spp.

Table 2. Suitability of nitrogen source for the production of DBP by *Streptomyces albidoflavus* MTCC 3662

Nitrogen sources (≃ 0.32 N g/l)	Yield (µg/ml)
Ammonium nitrate	125±2.24
Ammonium chloride	140±1.58
Ammonium dihydrogen phosphate	110±2.24
Ammonium sulphate	145±1.58
Arginine	290±2.24
Asparagine	190±2.74
Di-ammonium hydrogen orthophosphate	145±2.24
Potassium nitrate	145±1.58
Sodium nitrate	175±3.16
Tyrosine	175±3.16
Urea	75±2.24
Control	110±2.74-

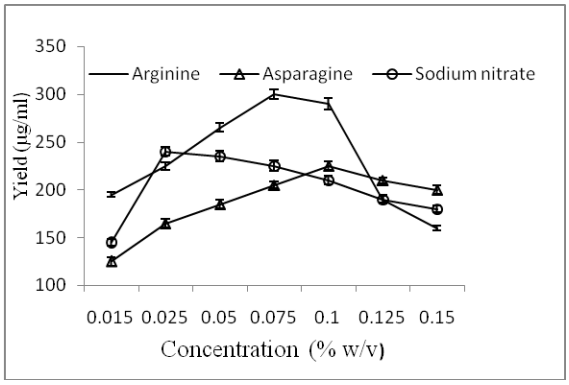

Figure 2. Effect of nitrogen on the production of DBP by *Streptomyces albidoflavus* MTCC 3662

3.3. Effect of Phosphate

The limitation of phosphate can regulate the metabolic rate; hence, the source and amount were varied. Thus, the production was maximum at 0.05% KH_2PO_4 (Figure 3). In transcriptional regulation the involvement of phosphate is well established for biosynthesis of secondary metabolites (Reeve and Baumberg, 1998). Phosphate limitations were also observed (Kishimoto *et al*., 1996). Data of this experiment could well corroborate with the observation of Ochi *et al*. (1988) and Ogawa *et al*. (1998).

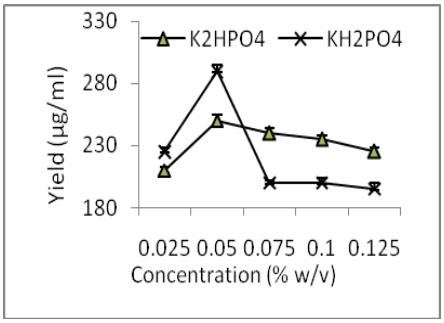

Figure 3. Effect of phosphate on the production of DBP by *Streptomyces albidoflavus* MTCC 3662.

3.4. Effect of Chloride

Apparently, chlorine may not play an important nutritional role, but its limitation concerns. Sometimes, range of osmotic role for growth is quite narrow and varies with the habitational limitations of the species; hence, the adjustment of NaCl concentration was recommended (Stanbury *et al*., 1997). Commonly used chlorine salts, like NaCl and KCl, were used to determine their optimum concentration and was found 0.15% NaCl (Figure 4). This is closely related to the reported range of 0.1% (Kojiri *et al*., 1992) to 0.25% (Harindran *et al*., 1999). Even though KCl was also used by several workers (Tunac *et al*., 1985; Nakamura *et al*., 1986) the present experiment did not support.

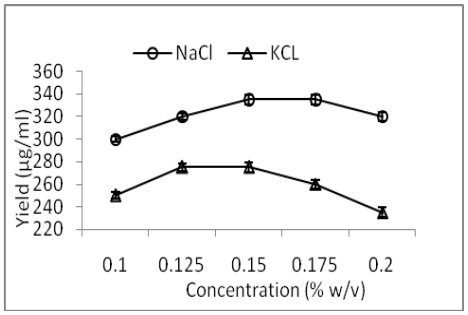

Figure 4. Effect of chloride on the production of DBP by *Streptomyces albidoflavus* MTCC 3662

3.5. Effect of Sulphate

Sulphur is a component part of protein, enzyme and coenzyme A. Quite often, adequate synthesis of enzyme depends on the availability of sulphur. Therefore, experiment was conducted with different concentrations of $MgSO_4$ as the source of sulphur and 0.03% was found best for the yield of antimetabolite (Figure 5). Role of $MgSO_4$ was reported for production of antimicrobials by *Streptomyces* spp. and optimized at 0.03% (Ochi *et al*., 1988) and 0.05% (Ogawa *et al*., 1998).

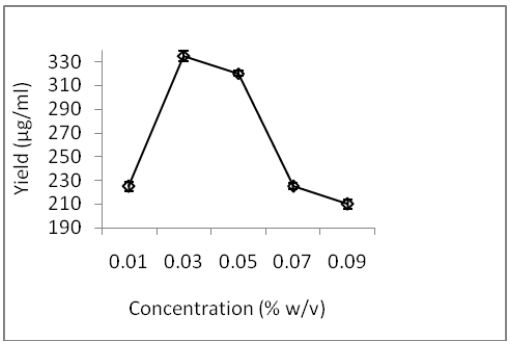

Figure 5. Effect of $MgSO_4$ on the production of DBP by *Streptomyces albidoflavus* MTCC 3662

3.6. Effect of Trace Element

Solubility and ionization often regarded as important factors for influencing the yield of secondary metabolites. Martin and McDaniel (1977) suggested that the metal ions probably activate the enzymes involved in synthetic steps of secondary metabolism. The salts of manganese, iron and zinc were found to play critical role in secondary metabolism (Weinberg, 1970). While studying the effect

of trace salts with *Streptomyces albidoflavus* 321.2 medium was prepared out using triple distilled water and tap water. The results showed that Mn^{++} (as $MnSO_4$, 0.001g/l) alone could enhance yield. But the yield was further enhanced when all the trace salts were used together (Table 3). H aque *et al*. (1995) observed the importance of manganese and zinc for the production of antimicrobial compound from *Streptomyces.*

Table 3. Effect of trace elements for the production of DBP by *Streptomyces albidoflavus* MTCC 3662

Trace salts	Concentration (%)	Yield (µg/ml)
Fe ($FeSO_4$)	0.01	265±3.54
	0.001[a]	290±2.74
	0.0001	250±2.24
Cu ($CuSO_4$)	0.001	135±4.48
	0.0001	175±4.48
	0.00001[b]	225±3.16
Mn ($MnSO_4$)	0.01	240±5.24
	0.001[c]	400±5.70
	0.0001	265±3.87
Zn ($ZnSO_4$)	0.001	290±4.48
	0.0001[d]	320±4.18
	0.00001	290±3.16
Tap water	--	265±4.18
a+b+c+d	--	450±6.12
Control (as recommended in basal medium)	--	335±5.24

3.7. Effect of Supplement

Supplementation of synthetic medium with natural compounds increases nutrient support viz; vitamins that commonly play notable role in cell metabolism. While checking the effect of supplementation, the yield was enhanced only with yeast extract (0.001%) and in combinations (Table 4). The role of yeast extract for the production of antimicrobial compound from *Streptomyces* was found both stimulatory (Haque *et al*., 1995) and inhibitory (Raytapadar and Paul, 2001).

Table 4. Effect of supplement for the production of DBP by *Streptomyces albidoflavus* MTCC 3662

Supplement	Concentration (%)	Yield (µg/ml)
Casein hydrolysate	0.1	240± 2.74
	0.01	290± 2.24
	0.001[a]	450± 4.18
	0.0001	450±5.24
Jaggary	0.1	265± 3.87
	0.01	335±4.18
	0.001[b]	450±4.48
	0.0001	450±5.24
Yeast extract	0.1	225±3.16
	0.01	240±4.18
	0 .001[c]	500±6.32
	0.0001	500±6.12
Control (without supplement)	_	450±5.70
a+b+c+d	-	550±6.32

3.8. Effect of Surfactant

With the use of surfactants, yield enhancement was observed only at 0.2 µg/ml of Tween-80 (Table 5). Surface acting agents are known to increase the membrane permeability and to influence in flow and out flow for biosynthesis (Abbott and Gledhill, 1971). Medium supplemented with fatty acids and detergents were found to increase yield of antimicrobial compound by *Streptomyces* (Mouslim *et al*., 1997).

Table 5. Effect of surfactants for the production of DBP by *Streptomyces albidoflavus* MTCC 3662

Surfectants	Concentration (µg/ml)	Yield (µg/ml)
Teepol	0.02	550±6.32
	0.2	500±4.48
	2	90±2.24
SDS	0.02	350 ±3.87
	0.2	225±4.18
	2	100±3.16
EDTA	0.02	500 ±6.12
	0.2	350±3.16
	2	135±2.74
Tween-80	0.02	550±5.24
	0.2	600±6.32
	2	225±3.16
Control (without surfectant)	-	550±5.7

4. Conclusions

The synthesis of secondary metabolite in excess is controlled by the genetic makeup and its fullest expression is possible only by the identification of limiting conditions. However, at least part of what can be substantiated by the above experimental results from using *Streptomyces albidoflavus* MTCC 3662 as producer, and the yield is increased to 2.4 fold, at its wild state of the producer.

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In vivo Assay for Antagonistic Potential of Fungal Isolates against Faba bean (*Vicia faba* L.) Chocolate Spot *(Botrytis fabae* Sard.*)*

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Abstract

Due to its high protein content, faba bean (*Vicia fabae* L.) leaves harbor many microorganisms besides *Botrytis fabae*. The objective of this study is to explore fungal isolates residing on faba bean leaves and evaluate their antagonistic potential against *B. fabae*. For this matter, 236 leaf samples were collected from different districts of West Hararghe and Bale zones. Out of which 72 fungal species were isolated and evaluated for their *in vivo* biocontrol potential against chocolate spot (*B. fabae* Sard.). The *in vivo* assay was conducted in two stages where detached leaf test and intact leaf test was involved. Significant difference (p<0.05) resulted among fungal isolates to affect incubation period (IP) and disease severity (DS) on local and Shalo cultivars on detached leaf. Isolates GO2-3, GB6-3, S16-2, A12-1 and 52-BT resulted incubation period of 3.7 - 4.7 days, where it was 2 days on untreated control of both cultivars. Lower disease severity was recorded from the leaf treated by GO2-3 and S16-2 on local and GO3-2 on Shalo based on 1-4 rating scale. On intact plant, significant difference (p<0.05) among fungal isolates was resulted to affect IP, diseases incidence (DI) and DS. Higher IP was recorded from isolates GB6-3 (3.3), S16-2 (3), and GO3-2 (3 days) on local. GO3-2 showed better reduction (66.7 %) of chocolate spot incidence on Obse compared to the control (100%). Isolates S16-2, GO3-2 and GB6-3 resulted lower disease severity (percent severity index) of 35.6-51.1% as compared to control (73.3-84.4%) on the three cultivars.

Keywords: Faba Bean, *Botrytis fabae*, Antagonistic Fungi, Biocontrol, *Trichoderma* Spp.

1. Introduction

Faba bean (*Vicia faba* L.) is a food and feed legume of great socio-economic importance and is one of the earliest domesticated food legumes in the world, probably in the late Neolithic period (Metayer, 2004). Faba bean ranks sixth in production among the legumes grown in the world. China has been the main producing country, followed by Ethiopia, Egypt, Italy, and Morocco (Salunkhe and Kadam, 1989). Even though Ethiopia is the world's second largest producer of faba bean, its share is only 6.96% of world production and 40.5% within Africa (Chopra *et al*., 1989). The average yield of this crop under small-holder farmers ranges from 1.0 to 1.2 t ha^{-1} (Agegnehu *et al.,* 2006), while world average grain yield of faba bean is around 1.8 t ha^{-1} (ICARDA, 2008). In the Ethiopia highlands, faba bean is one of the most important food crops. It is a source of cash to the farmers and foreign currency to the country. The growing importance of faba bean as an export crop in Ethiopia has led to a renewed interest by farmers to increase the area under production (Samuel *et al.,* 2008). However, the productivity of faba bean in Ethiopia is far below its potential due to a number of factors. The biological limitations include inherently low grain yielding potential of the indigenous cultivars and susceptibility to biotic and abiotic stresses (Mussa *et al.,* 2008). Diseases, chocolate spot (*Botrytis fabae* Sard.), rust (*Uromyces Vicia fabae*), and black root rot (*Fusarium solani*) contribute to the low productivity of the crop. Chocolate spot is considered to be the most important and destructive in Ethiopia causing the yield loss of up to 61% on susceptible cultivars (Dereje and Beniwal, 1987).

Currently, there is an urgent need to improve faba bean yield, since this crop remains an important part of Ethiopian diet. Although synthetic chemicals are available as better option, Products from microbes are relatively broad spectrum, bio-efficacious, economical, and environmentally safe and can be ideal candidates for use as bio-pesticides (Macias *et al.,* 1997). Among these, antagonistic microbioagents from soil and/or phylloplane of plants have been reported to show activity against wide array of plant pathogenic fungi (Reddy, 2000). Therefore, controlling *B. fabae* by biocontrol agents seems to be better and preferred than the chemical control (Mahmoud

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et al., 2004). Little research, conducted in Ethiopia for the control of *B. fabae*, indicated high potential of local microbial agents (Samuel, 2008). There is a dire need of exploring different areas for suitable and highly effective microbes for management of chocolate spot. Therefore, this work is proposed with the objectives of identifying the potential antagonistic microorganisms associated with the phylloplane of faba bean and evaluates the effect of potential antagonistic microorganisms against chocolate spot.

2. Materials and Methods

2.1. Collection of Faba Bean Leaf Samples

Two hundred forty samples of healthy looking faba bean leaves were collected from faba bean plants showing chocolate spot disease for exploring the resident fungal isolates (Table 1). Ten fields 5–10 km apart from each districts were visited and from each field 3-4 healthy looking plants were randomly selected, and four healthy looking leaves were detached from each plant. Similarly, Faba bean leaves naturally infected by chocolate spot were collected for isolation of *Botrytis fabae*.

Table 1. Faba bean leaf sample collection from major faba bean producing districts of west Hararghe and Bale zones, Oromia.

Zone	District	Altitude (masl)	No. of sample
Bale	Sinana	2361 - 2396	28
	Goro	1981- 2332	28
	Agarfa	2404 - 2501	28
	Goba	2430 - 2606	40
	Gassera	2369-2422	36
West Hararghe	Bedeno	2308-2605	40
	Deder	2401-2737	40

2.2. Isolation of Botrytis fabae and Resident Fungal Isolates from Phylloplane

Botrytis fabae was isolated from faba bean leaves naturally infected by chocolate spot. Leaves were surface disinfected with 1% sodium hypochlorite for 2 min and rinsed in two changes of sterile water, placed on Potato Dextrose Agar (PDA), incubated at 20°C for 7 da ys (Haggag *et al*., 2006), and purified by repeated sub-culturing. Likewise, antagonistic fungal isolates residing on faba bean leaves were isolated on PDA media. The collected healthy looking leaf samples were washed in two changes of sterile water for 10 minutes each and macerated using mortar and pestle. The suspension was diluted at 10^{-2}, poured on PDA and incubated at 25^{o}C for 7 days. All visible fungal colonies were isolated, purified, coded and stored at 4^{o}C. The fungal isolates which were later found effective were identified.

2.3. Detached Leaf Test

Fifteen antagonistic fungal isolates out of 72 were finally evaluated for antagonistic potential against chocolate spot on detached leaves. Leaves were prepared by detaching apparently healthy looking leaves from faba bean plants grown at open fields of Haramaya University (HU) research site. HU is located at 42^{0} 30’ E longitude and 9^{0} 26’ N latitude elevated at 1980 masl. It receives 780 mm total annual rain fall and minimum and maximum temperature of 1.4^{0}C and 23.4^{0}C, respectively. The faba bean varieties Shalo (EH011-22-1) and Bale local were arranged in Randomized Complete Block Design (RCBD) in this study. Fully expanded leaflets of similar age group were detached from 6 weeks old faba bean plants, from the middle nodes of the two varieties. Leaflets were surface disinfected by 1% sodium hypochlorite for 2 m in, and subsequently rinsed with distilled sterile water and allowed to dry on sterile filter paper. Sterile filter paper was put in side the petri dishes and moistened by distilled sterile water. Sterile bent glass rod was put on the filter paper and leaves were put on the glass rod to serve as moist chamber. *B. fabae* and antagonistic fungal isolate spore suspensions were prepared from 10 da ys old culture. The spore concentration was adjusted to 2.5 x 10^5 spores /ml by using a hemacytometer (Mohammed *et al.*, 1994). One drop (20 µl) of the antagonistic fungal spore suspension was placed near the midrib of the leaves. The Petri plates were incubated at 20 ^{0}C for 36 hrs. Then, a drop of *B. fabae* spore suspension, containing 2.5 x 10^5 spores /ml was added to the midrib, where the drop of the antagonistic fungal spore suspension was placed and incubated at 20 ^{0}C. Plate containing detached leaf inoculated only with *B. fabae* alone was used as control. The study was conducted in three replications arranged in RCBD. The disease development was rated using a 1 -4 scale (ICARDA, 1986) where 1 = highly resistant, no infection or very small flecks (1-25% necrosis); 2 = resistant, necrotic flecks with few small lesions (26-50% necrosis), and very poor sporulation; 3 = moderately resistant, medium coalesced lesions (51-75% necrosis) with intermediate sporulation; and 4 = susceptible, large coalesced lesions (76-100% necrosis) with abundant sporulation.

2.4. Intact Plant Test

Nine fungal isolates which showed promising results in detached leaf tests were further evaluated in intact plant test (greenhouse). Three faba bean varieties, Obse (EH95073-1), Shalo (EH011-22-1) and Bale local were arranged in RCBD using three replications. Seeds were surface disinfected in 1% sodium hypochlorite for 10 min followed by washing in three changes of distilled sterile water. The spore suspension of both *B. fabae* and the fungal isolates were prepared in the same way as in section 3.6 above. Six weeks old faba bean plants grown in greenhouse using 23.5 cm diameter plastic pots (5 plants/pot) field with sand, manure and compost in 1:2:3 ratio were sprayed with 20 m l/plant/pot of each fungal isolates at a concentration of 2.5x10^5 spores/ml (Mohammed *et al.*, 1994). Inoculated pots were covered with moistened plastic bags for 24 hr to increase the relative humidity of the environment to favor the development of sprayed fungi. After 2 days of incubation, plants were inoculated by 20 ml/plant/pot of *B. fabae* spore suspension, containing 2.5x10^5spores/ml. Pots sprayed with 20 m l/plant/pot *B. fabae* spore suspension alone was used as control. Thereafter, each pot was

covered with moist plastic bags for 24 hr to maintain high relative humidity (RH) of the environment. The temperature and the RH of the greenhouse during the study period were in a range of 19-22^{0}C and 88-91%, respectively. The disease development was rated using 1-9 scale, where, 1= No disease symptoms or very small specks; 3= few small discrete lesions; 5= some coalesced lesions with some defoliation; 7= large coalesced sporulating lesions, 50% defoliation and some dead plant; and 9= Extensive lesions on leaves, stems and pods, severe defoliation, heavy sporulation, stem girdling, blackening and death of more than 80% of plants (Bernier *et al*., 1993). The disease data recorded based on scoring scale mentioned above was converted to percentage severity index (PSI) according to Wheeler (1969):

$$PSI = \frac{\text{Sum of Numerical Ratings X 100}}{\text{Number of Plants Scored X Maximum Score on Scale}}$$

2.5. Data Analysis

Data on incubation period (days), disease incidence (%) and severity (%) were analyzed using ANOVA SAS procedure (SAS, 2002), to know the effect of fungal isolates on the growth of the pathogen and development of chocolate spot. Least significant difference (LSD) value was used to separate the treatment means.

3. Results

3.1. Detached leaf test

Incubation period: Significant difference ($p<0.05$) were obtained among fungal isolates in affecting the incubation period. Out of the total isolates evaluated, five of them were better in increasing the incubation period. Isolates GO2-3 (*T. harzianum*), GB6-3 (*T. harzianum*), S16-2 (*T. polysporum*), A12-1 (*T. oblongisporum*) and 52-BT (*T. longibrachiatum*) prolonged the incubation period to 4.7, 4.7, 4.7, 4 and 4 days on local cultivars and 3.7, 4.3, 4.3, 4 and 4.3 days on Shalo variety, where as incubation period on control was 2 days on both varieties (Table 2). Alison and Mansfield (1984) in their experiment on onion bulb scales and detached leaves for their response to the development of *Botrytis* spp. showed that *B. squamosa* developed spreading lesion within three days of inoculation. *B. allii* and *B. cinerea* also developed spreading lesions within five days of inoculation and *B. fabae* was also produced limited lesions five days after inoculation. In the current study, the first visible symptom of *B. fabae* was observed within two to five days of inoculation depending on the type of fungal antagonists inoculated with the pathogen and within two days of inoculation on control. Bouhassan *et al.* (2004) in their experiment to screen faba bean genotypes to chocolate spot resistance, reported that small lesions characteristic of chocolate spot appeared six to eight hours after inoculation. As they enlarged with time, these small spots fused to form larger lesions, the severity of which varied according to lines. The mean of the scores indicated that the discrimination among the lines was significant three days after inoculation based on the lesion visual score and five days after inoculation based on the lesion diameter.

Table 2. Effect of antagonistic fungal isolates on incubation period (days) of faba bean chocolate spot on two varieties.

Isolate	Fungal species	Local	Shalo [1]
Go2-3	*Trichoderma harzianum*	4.67^{a}	3.67^{ab}
Gb6-3	*Trichoderma harzianum*	4.67^{a}	4.33^{a}
S16-2	*Trichoderma polysporum*	4.67^{a}	4.33^{a}
A12-1	*Trichoderma oblongisporum*	4.00^{ab}	4.00^{a}
52-BT	*Trichoderma longibrachiatum*	4.00^{ab}	4.33^{a}
S11	*Trichoderma hamatum*	3.33^{bc}	3.67^{ab}
117-2T	*Trichoderma longibrachiatum*	3.00^{cd}	2.33^{c}
Go3-2	*Trichoderma gamsi*	3.00^{cd}	3.67^{ab}
Gb25-3	*Trichoderma virens*	2.67^{cd}	2.33^{c}
Gb15-2	*Trichoderma spirale*	2.67^{cde}	2.33^{c}
2An	*Trichoderma koningii*	2.33^{de}	2.67^{bc}
Ga3-2	*Trichoderma longibrachiatum*	2.33^{de}	2.67^{bc}
2A-17	*Trichoderma koningii*	2.00^{e}	2.67^{bc}
Gb25-1	*Trichoderma citrinoviride*	2.00^{e}	2.67^{bc}
Ga3-3	*Trichoderma ovalisporum*	2.00^{e}	2.00^{c}
Control		2.00^{e}	2.00^{c}
LSD		0.90	1.13
CV (%)		17.52	21.81

Means in the same column with the same letter are not statistically different at $p\leq0.05$. [1] Mean incubation period for chocolate spot symptom development (mean of the three replications).

Diseases severity: Significant difference ($P<0.05$) occurred among fungal isolates on both Shalo and local faba bean cultivars in reducing the disease severity. On both varieties the lowest disease severity was recorded from the leaf treated by Go2-3 (*T. harzianum*) with disease score of 1, followed by S16-2 (*T. polysporum*) and Go3-2 (*T. gamsi*) with disease severity of 1 and 1.33 on local and Shalo varieties, respectively. While on control the disease severity was 2 and 2.33 on local and Shalo, respectively (Table 3). Omar *et al.* (1986) in their research to explore the effect of virus infection on developmen of *Botrytis* lesion by detached leaf technique, found that chocolate spot caused by *B. fabae* developed very well on both virus free and virus infected leaves. Lesion development was most rapid and extensive and sporulation most pronounced on the oldest leaf with lession development rate of up to 1.9 mm/day on plants artificially infected with spore concentration of 2 x 10^5 spores/ml. Like wise, they recorded a percent severity index (PSI) of up to 21.1% and 54.2% 3 and 9 days after inoculation, respectively. In the current study more or less comparable results were obtained. Samuel *et al.* (2009) reported that, out of the total 20 *Bacillus* isolates screened most of the isolates reduced development of *B. fabae* on detached faba bean leaves. Most of the isolates limited

chocolate spot expansion to 1-2.5 in 1-5 scoring scale while the development of the disease reached 4.5 on local cultivar based on the same scale.

Table 3. *In vivo* effect of fungal isolates on faba bean chocolate spot severity using detached leaf technique on two varieties.

Isolate	Fungal species	Local	Shalo [1]
S16-2	*Trichoderma polysporum*	1.00^{c}	1.33^{bc}
2An	*Trichoderma koningii*	1.00^{c}	1.67^{abc}
Go3-2	*Trichoderma gamsi*	1.00^{c}	1.33^{bc}
Go2-3	*Trichoderma harzianum*	1.00^{c}	1.00^{bc}
A12-1	*Trichoderma oblongisporum*	1.00^{c}	1.33^{bc}
Gb6-3	*Trichoderma harzianum*	1.33^{bc}	1.33^{bc}
2A-17	*Trichoderma koningii*	1.33^{bc}	1.00^{c}
S11	*Trichoderma hamatum*	1.33^{bc}	1.33^{bc}
52-BT	*Trichoderma longibrachiatum*	1.33^{bc}	1.33^{bc}
Ga3-2	*Trichoderma longibrachiatum*	1.67^{ab}	2.00^{ab}
Ga3-3	*Trichoderma ovalisporum*	1.67^{ab}	2.00^{ab}
Gb25-3	*Trichoderma virens*	1.67^{ab}	2.00^{ab}
117-2T	*Trichoderma longibrachiatum*	2.00^{a}	2.00^{ab}
Gb25-1	*Trichoderma citrinoviride*	2.00^{a}	2.00^{ab}
Gb15-2	*Trichoderma spirale*	2.00^{a}	2.00^{ab}
Control		2.00^{a}	2.33^{a}
LSD		0.64	0.68
CV (%)		26.19	25.12

Figures in the same column with the same letter are not statistically different at $p \leq 0.05$. [1] Mean disease severity based on 1-4 rating scale for detached leaf test (ICARDA, 1986) where 1 = highly resistant, no infection or very small flecks (1-25 % necrosis); 2 = resistant, necrotic flecks with few small lesions (26-50 % necrosis), and very poor sporulation; 3 = moderately resistant, medium coalesced lesions (51-75 % necrosis) with intermediate sporulation; and 4 = susceptible, Large coalesced lesions (76-100 % necrosis) with abundant sporulation.

3.2. Intact plant test

Incubation period: The bioagents varied in their potential to increase the incubation period of the chocolate spot in greenhouse. On local cultivar, significant difference ($p<0.05$) resulted among fungal isolates in their potential to increase the incubation period of *B. fabae*. Out of the total isolates evaluated, three of them were better in increasing the incubation period of chocolate spot. Isolates GB6-3 (*T. harzianum*), S16-2 (*T. polysporum*), and GO3-2 (*T. gamsi*) prolonged the incubation period to 3.33, 3 a nd 3 days, respectively (Table 4). The difference between bioagents in increasing the incubation period on Shalo and Obse varieties was not statistically significant. The incubation period on control pot was 2 (two) days after inoculation (Table 4).

Table 4. *In vivo* effect of antagonistic fungal isolates on incubation period (days) of faba bean chocolate spot in greenhouse condition on three varieties.

Isolate	Fungal species	Local	Shalo	Obse[1]
GB6-3	*Trichoderma harzianum*	3.33^{a}	3.33	3.33
GO3-2	*Trichoderma gamsi*	3.00^{b}	3.33	3.00
S16-2	*Trichoderma polysporum*	3.00^{b}	3.00	3.00
2A-17	*Trichoderma koningii*	2.00^{c}	3.33	3.67
52-BT	*Trichoderma longibrachiatum*	2.00^{c}	3.33	3.33
GO2-3	*Trichoderma harzianum*	2.00^{c}	3.00	3.33
A12-1	*Trichoderma oblongisporum*	2.00^{c}	3.00	3.00
S11	*Trichoderma hamatum*	2.00^{c}	3.00	3.00
2An	*Trichoderma koningii*	2.00 ^{c}	3.00	3.33
Control		2.00^{c}	3.00	3.00
CV (%)		7.82	11.65	12.76
LSD		0.31	NS	NS

Means in the same column designated with the same letter are not statistically different at $p \leq 0.05$. NS-not significant

[1] is Mean incubation period for chocolate spot on local, Shalo and Obse varieties from the three replications.

Rhaiem *et al.* (2002) reported chocolate spot disease symptoms on the leaves and stems of faba bean three days after inoculation, and 7 days after inoculation the susceptible check was already fully infected. On another experiment, El-Hendawy *et al.* (2010) reported the occurrence of chocolate spot lesion 24 hr after artificial inoculation *of B. fabae* in greenhouse. They found disease severity of more than 10, 25 and 65% at 24, 48 and 72 hr after inoculation, respectively.

Disease incidence: Significant difference ($P<0.05$) were observed among the antagonistic fungal isolates evaluated on all the three varieties in reducing chocolate spot incidence. Out of the tested isolates, three of them were effective in reducing the disease incidence, GB6-3 (*T. harzianum*), S16-2 (*T. polysporum*) GO3-2 (*T. gamsi*) showed better reduction 61.67, 59 a nd 66.67% of chocolate spot incidence on Obse as compared to control (100%) on all varieties, respectively. On plants treated with the rest isolates high percentage of disease incidence (79–100%) was recorded regardless of varieties. However 100% disease incidence was recorded from few pots of local and Shalo varieties treated with some bioagents and on control of all the three varieties (Table 5). High frequency of 100% chocolate spot incidence was recorded from Shalo and no 100 % disease incidence was recorded on Obse except from the control 12 days after inoculation. A number of antagonistic fungal isolates are observed to affect the incidence of plant diseases. Different

researchers reported the potential of the antagonists to reduce disease incidence. A report indicated that gladiolus corms dipped in the culture of *Trichoderma* and four species of *Gliocladium*, not only reduced the disease incidence but also supported better sprouting and yield of corms (Kohl *et al*., 1997; Tesfaye, 1998). The action of antagonistic fungal isolates is not limited to its effect after the symptom development, some antagonists showed their potential effect on spore germination. Elda and Kapt (1999) reported that isolates of *T. harzianum* produced protease in liquid culture medium and on the surface of Bean leaves and reduction in *B. cinerea* germination, and germ tube length.

Disease Severity: Significant difference was obtained among antagonistic fungal isolates ($P<0.05$) of different species for their potential to reduce Chocolate spot severity (Table 6). Out of the tested antagonistic fungal isolates, three isolates showed better performance in suppressing the disease expansion on the three varieties almost equally. Isolates S16-2 (*T. polysporum*), GO3-2 (*T. gamsi*) and GB6-3 (*T. harzianum*) are the best performing fungal isolates which were effective of all the tested isolates which showed percent lesion reduction of 39.47, 39.47 and 42.10%, on local, 57.89, 53.50 and 44.73% on Shalo, 47.47, 48.44 and 42.42% on Obse, respectively over the control from which the highest PSI was recorded. From control pots, PSI of 84.44%, 84.44% and 73.33% were recorded on local, Shalo and Obse varieties, respectively. PSI recorded from leaves treated by the three effective fungal bioagents were 48.89%, 46.67% and 42.22% from GB-6-3%, 51.11%, 39.26% and 37.78% from GO3-2 and 51.11%, 35.56% and 38.52% from S16-2 on local, Shalo and Obse varieties, respectively (Table 6). In the activity of biological control, micro-organisms action is not limited to direct influence on the target diseases, in addition to their direct effect they also enhance the resistance of the plants. A report by Benítez *et al.* (2004) indicates that *Trichoderma* strains are known to promote plant growth and plant defensive.

Table 5. *In vivo* effect of antagonistic fungal isolates on faba bean chocolate spot incidence (%) in greenhouse on three varieties.

Days after inoculation	Isolate	Fungal species	Shalo	Local	Obse [1]
4	GB6-3	*Trichoderma harzianum*	12.67^{d}	30.33^{b}	5.33^{e}
	S16-2	*Trichoderma polysporum*	20.67^{c}	67.33^{a}	8.00^{e}
	GO3-2	*Trichoderma gamsi*	24.33^{c}	56.00^{ab}	13.33^{d}
	GO2-3	*Trichoderma harzianum*	39.33^{b}	29.00^{b}	27.67^{bc}
	2A-17	*Trichoderma koningii*	40.33^{b}	46.00^{ab}	29.67^{b}
	52-BT	*Trichoderma longibrachiatum*	40.67^{b}	42.00^{ab}	24.33^{c}
	A12-1	*Trichoderma oblongisporum*	43.00^{b}	64.67^{a}	29.33^{b}
	S11	*Trichoderma hamatum*	43.33^{b}	62.33^{ab}	28.67^{bc}
	2An	*Trichoderma koningii*	44.00^{b}	53.17^{ab}	27.67^{bc}
	Control		53.67^{a}	64.33^{a}	45.67^{a}
	LSD		33.7	7.1	4.6
	CV(%)		11.45	38.38	1.20
8	GB6-3	*Trichoderma harzianum*	53.00^{d}	60.00^{ab}	32.00^{d}
	S16-2	*Trichoderma polysporum*	61.67^{c}	92.17^{a}	30.67^{d}
	GO3-2	*Trichoderma gamsi*	57.33^{cd}	83.33^{a}	34.67^{d}
	GO2-3	*Trichoderma harzianum*	79.33^{b}	46.61^{b}	58.67^{b}
	2A-17	*Trichoderma koningii*	81.00^{ab}	77.33^{ab}	60.00^{b}
	52-BT	*Trichoderma longibrachiatum*	78.97^{b}	67.67^{ab}	51.67^{c}
	A12-1	*Trichoderma oblongisporum*	83.33^{ab}	89.00^{a}	61.67^{b}
	S11	*Trichoderma hamatum*	81.00^{ab}	90.67^{a}	61.33^{b}
	2An	*Trichoderma koningii*	81.33^{ab}	81.15^{ab}	57.67^{bc}
	Control		86.56^{a}	91.00^{a}	82.00^{a}
	LSD		36	6.8	6.2
	CV(%)		5.40	27.17	6.83
12	GB6-3	*Trichoderma harzianum*	84.00^{c}	87.33^{ab}	61.67^{de}
	S16-2	*Trichoderma polysporum*	91.67^{b}	100.00^{a}	59.00^{e}
	GO3-2	*Trichoderma gamsi*	91.00^{b}	96.67^{ab}	66.67^{d}
	GO2-3	*Trichoderma harzianum*	98.00^{a}	79.67^{b}	82.00^{bc}
	2A-17	*Trichoderma koningii*	100.00^{a}	93.67^{ab}	81.33^{bc}
	52-BT	*Trichoderma longibrachiatum*	97.00^{a}	87.33^{ab}	75.67^{c}
	A12-1	*Trichoderma oblongisporum*	100.00^{a}	100.00^{a}	83.67^{b}
	S11	*Trichoderma hamatum*	100.00^{a}	100.00^{a}	80.67^{bc}
	2An	*Trichoderma koningii*	100.00^{a}	97.00^{ab}	80.67^{bc}
	Control		100.00^{a}	100.00^{a}	100.00^{a}
	LSD		17.9	3.3	7.4
	CV(%)		2.04	11.17	5.63

Means in the same column with the same letter are not statistically different at $p\leq0.05$,

Mean diseases incidence of chocolate spot on the three varieties from the three replications

Table 6. *In vivo* effect antagonistic fungal isolates on faba bean chocolate spot percent severity index in greenhouse on three varieties.

Isolate	Fungal species	Local	Shalo	Obse[1]
GB6-3	*Trichoderma harzianum*	48.89^{c}	46.67^{d}	42.22^{c}
GO3-2	*Trichoderma gamsi*	51.11^{c}	39.26^{e}	37.78^{d}
S16-2	*Trichoderma polysporum*	51.11^{c}	35.56^{e}	38.52^{d}
52-BT	*Trichoderma longibrachiatum*	75.56^{b}	59.26^{c}	61.48^{b}
GO2-3	*Trichoderma harzianum*	78.52^{ab}	80.74^{ab}	61.48^{b}
A12-1	*Trichoderma oblongisporum*	82.22^{a}	80.00^{ab}	60.74^{b}
2An	*Trichoderma koningii*	82.22^{a}	83.70^{a}	61.48^{b}
S11	*Trichoderma hamatum*	82.96^{a}	78.52^{b}	60.74^{b}
2A-17	*Trichoderma koningii*	82.96^{a}	81.48^{ab}	61.48^{b}
Control		84.44^{a}	84.44^{a}	73.33^{a}
LSD		6.66	4.79	1.83
CV(%)		5.43	4.20	1.91

Means in the same column designated with the same letter are not statistically different at $p\leq0.05$. [1] Mean PSI of chocolate spot on three faba bean varieties of three replications.

4. Discussion

In Ethiopia, this type of study is at its infant stage but, little research conducted has indicated high potential of local microbial agents (Samuel *et al.*, 2009). Fifteen fungal species from phylloplane of faba bean leaves were tested *in vivo*, against Chocolate spot in detached leaf test. Out of which nine isolates were promoted to intact plant test in greenhouse. Finally, three of them were found to be effective against chocolate spot. Samuel *et al.* (2009) tested a number of *Bacillus* and *Trichoderma* species and found the result complementing to ours. Similarly, Lo and Lin (2002) reported the potential of *Trichoderma* spp. to enhance plant growth in addition to its disease control potential. The isolates had different potential of controlling the disease on varieties having different level of resistance against the disease Samuel *et al.* (2009), the finding from this study have also confirmed that the resistance level of the varieties have direct influence on the efficacy of isolates. In Egypt, damping off disease incidence was highly reduced by application of *Trichoderma* species. Very low disease incidence of 9-19% and 2.5-7.5% was recorded with application of *Trichoderma* spp. compared to control where 48.5 and 55.8% was recorded at pre- and post-emergence stages, respectively (Abd-El-Khair *et al.*, 2010). Finding from other study revealed that seed inoculation and foliar spray of *Trichoderma* spp. significantly reduced incidence and severity of chocolate spot to the level it can be comparable with fungicides (Saber *et al.*, 2009). Similar result was found in our study, where different *Trichoderma* spp. was tested and three of them showed better performance in reducing chocolate spot incidence. *T. gamsi* gave the highest disease incidence reduction of 66.67%, followed by *T. harzianum* (61.67%) and *T. polysporum* (59%), where 100% incidence was recorded on control which is in agreement with the above finding. Biocontrol agents differ in their disease control potential when applied individually or in combination. Poornima, (2011) reported that *Trichoderma* spp. and *Pseudomonas* spp. showed 62.6% and 36.1% disease control, respectively when applied individually, but with application of *Trichoderma* spp. + *Pseudomonas* spp. the disease control was 50.6%. In the current study, three individually applied isolates; *T. harzianum*, *T. gamsi* and *T. polysporum* lowered disease severity to 42.22-51.11% as compared to the control (73.73-84.44%) on the three varieties. These isolates also affected the incubation period of chocolate spot. Haggag *et al.* (2006), reported that *Talaromyces flavus* and *Trichoderma harzianum* reduced brown spot disease severity on Faba bean. According to this study, lower disease severity of 1.2-6.4% and 2.4-12.5%, was recorded from plants treated with T. *flavus* and *T. harzianum,* respectively, while disease severity on control pot was 32.6%, 50 days after planting under artificial inoculation condition in greenhouse.

5. Conclusion

This study was undertaken to investigate the biocontrol potential on local micro-biota of fungi. A number of fungal isolates was tested against chocolate spot both on detached leaf and intact leaf tests. Almost all of the tested isolates showed biocontrol potential against chocolate spot with varying degrees. But, finally three fungal isolates (*Trichoderma* spp.) was found effective on detached leaf and intact plant tests. Even if found good result from this study, we recommend further study for the expansion and commercialization of these isolates.

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"Vinegar" as Anti-bacterial Biofilm formed by *Streptococcus pyogenes* Isolated from Recurrent Tonsillitis Patients, *in vitro*

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Abstract

Failure of antibiotic treatment in eradication of bacterial tonsillitis induced us to postulate presence of "bacterial biofilms" attached to living tissues that can be considered as an etiologic factor, among others. The knowledge about biofilm existence supports a new concept to explain chronic infections. Tonsillectomy is often the required choice as a consequence of frequent tonsillitis during the previous years, subsequently a novel studies propose using of non-antibiotic and non-surgical modalities for eradication of biofilm-related tonsillitis. In our designed study we used "vinegar" as anti-biofilm agent due to its antimicrobial effect. Vinegar is a sour liquid composed mainly of acetic acid, which has an important role in disruption of biofilm aggregations. This study has been undertaken to determine biofilm production ability by *Streptococcus pyogenes* isolated from tonsillitis patients and to evaluate effectiveness of different types of vinegar for eradication of Streptococcal-tonsillar biofilm, *in vitro*. Twenty nine isolates of *S. pyogenes* belonged to 19 patients, were tested for biofilm production by using Microtiter-plate method. Also, Modified Microtiter-plate method was conducted to study the effect of different vinegar types (Date, Apple, and Grape) as anti-biofilm, *in vitro*. Out of 75.9% produced biofilm according to depending biofilm criteria, while 24.1% were non-biofilm producers. Biofilm producer isolates distributed into 12(54.5%) and 10(45.5%) which was detected on tonsil surface and crypts respectively. Concerning the anti-biofilm activity, our results demonstrated that types of vinegar eradicated biofilm by (100%), (95.5%), and (90.9%) for Date, Apple, and Grape vinegar respectively in compare with distilled water as negative control. It was concluded that there was no significant differences in biofilm production between *S. pyogenes* isolated from tonsil surface and crypts. Furthermore, our study concluded that the three types of vinegar eradicated streptococcal biofilm remarkably, but Date vinegar was the best for eradication of streptococcal biofilm *in vitro*.

Keywords: Tonsillitis, *Streptococcus pyogenes*, Biofilm eradication, Vinegar**.**

1. Introduction

Bacteria that attach to surfaces aggregate in an extracellular polymeric matrix of their own synthesis or host origin to form biofilms. These bacteria are resistant to host defense mechanisms and antibiotics (Fux *et al*., 2005), but they can be released and cause acute infections. Antibiotic therapy can improve symptoms caused by bacteria that are released from biofilms, but cannot eliminate biofilms. For this reason, bacterial biofilms cause recurrent infections, until they are removed completely using surgical methods (Woo *et al*., 2012). Biofilms are currently considered to be an important cause of chronic infection. Several studies have reported the presence of biofilms in patients with recurrent tonsillitis (Chole and Faddis, 2003; Al-Marzrou and Al-Khattaf, 2008). Several bacterial species are able to develop a b iofilm, at the same time considered as important frequent pathogen for tonsillitis. For example *Streptococcus pyogenes*, *Haemophilus influenzae*, *Pseudomonas aeruginosa*, *Streptococcus pneumoniae* and *Staphylococcus aureus* (Post *et al*., 2004; Wang *et al*., 2005; Galli *et al*., 2007). They invade tonsil crypts and proliferate, causing an acute inflammatory reaction and pus formed in the crypts. The infection occasionally extends to adjacent tissues. So, tonsillectomy is advised after at least five repeated attacks of tonsillitis per year (Woo *et al*., 2012). Additionally, biofilms can directly colonize mucosal tissues, producing chronic and/or recurrent infections that are resistant to all types of antibiotic treatment (Galli *et al*., 2007). The upper airways seem to be at high risk for this type of colonization since evidence has been documented in the nasal and sinus mucosa of subjects with chronic hyperplastic sinusitis (Perloff and Palmer, 2005), and in the tonsillar crypts (Chole and Faddis, 2003).

Over the years, tonsillectomy remains the golden step for treatment. In spite of postoperative complications, a novel studies suggest using of non-antibiotic and non-

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surgical modalities for eradication of biofilm-related tonsillitis (Smith *et al*., 2011). Such as using of chemicals, surfactants (Suga and Igarashi, 2012) and other natural compounds (Hannig *et al*., 2009). Among these material, is vinegar which is a sour liquid comprised mainly of acetic acid, typically 4-18% acetic acid by mass, which is prepared in households by the fermentation of many fruits. This solution is also commercially available, it is cheap and easily found in markets. There are various studies which support the antimicrobial effects of vinegar (Nascimento *et al*., 2003). Vinegar has also been shown to be effective in the prevention and control of microbial contamination in intra-canal treatment of apical periodontitis in teeth (Estrela *et al*., 2004). Also, it has been used as removing agent for *Candida albicans* biofilm from Acrylic Resin Plates. The amount of acetic acid used as vinegar on a worldwide scale is not large, but is by far the oldest and best-known application (Jafari *et al*., 2012).

The main goals of the present study were to determine biofilm production ability by *Streptococcus pyogenes* isolated from infected tonsil surface and crypts, and it's relation with recurrent infection. In addition to evaluate the effectiveness of different types of vinegar (commercially available as table vinegar) for eradication of Streptococcal biofilm isolated from recurrent tonsillitis patients, *in vitro*.

2. Materials and Methods

2.1. Specimens Collection and Bacterial Isolates

A total of 29 bacterial isolates of *Streptococcus pyogenes* were isolated from infected tonsils after tonsillectomy for 19 patients with recurrent tonsillitis were employed for this study. The ranges of patients' age group were from 4 to 14 years; 12 of them were female and 7 were male. Those were with a clinical history of at least five to six times of tonsillitis per year. Full informative history had been taken directly from patients and the information was arranged in detailed formula sheet with surgeon assistance. All patients subjected to tonsillectomy and the presented study extended from September, 2012 to January, 2013. Sixteen isolates of *S. pyogenes* were identified on the surface of tonsils, while 13 isolates were identified in the crypts of tonsils. Bacterial isolates of *S. pyogenes* were identified by standard methods. In brief, depending on the colonial morphology and β-hemolytic activity on 5% blood agar medium. In addition to some of biochemical tests specially bacitracin discs (0.04 units, Becton, Dickinson and company spark, MD (TAXO) BD, USA) were used for identification of *S. pyogenes* (Goldman and Green, 2009). Isolated bacteria were maintained for intermediate storage in brain heart infusion broth with 20% glycerol in -20°C (Baron *et al*., 1994).

2.2. Microtiter Plate-Biofilm Production Assay

Quantitative determination of biofilm was made using microtiter plate adhesion assay in accordance with Stepanovic *et al.* (2000); Perez *et al*. (2011); Mulla and Revdiwala (2011) with some modifications. Briefly, three to five colonies were suspended with 5 ml of Trypticase soy broth (TSB) (HiMedia, India) and incubated for 24 hrs. Then, diluted and adjusted to 0.5 McFarland turbidity standards to reach 10^5 CFU/ml. An aliquot of 200 µL of diluted bacterial suspension with 0.25% glucose (BDH, England), was added to each well of 96-well flat-bottomed polystyrene microtitre plates (Div.Becton, Dickinson &Co. Oxnard ,California, USA) and incubated for 18-24 hrs. at 37°C. Media with suspended bacteria were then removed; the plates washed carefully 3-4 times with sterile distilled water and air-dried, then stained with 200 µL of 0.9% crystal violet (Fluka, Switzerland). After washing the dye, the attached bacteria solubilized with 95% ethanol and the optical density of the adherent biofilm was determined twice with a filter of 450/630nm in ELISA reader (STAT-FAX 3200, USA). In our experiment 200 µ L of TSB broth with 0.25% glucose were used as a negative control to obtain a background absorbance, which was then deducted from absorbance values obtained from the wells containing study isolates. All isolates were tested in triplicate.

The interpretation of biofilm production depended on the criteria used by Perez *et al*. (2011) depending on standard calculations laid down by Christensen *et al*. (1985) by which the study isolates were classified as the following : Non-producer, weak, moderate and strong producer; if the $OD_{450/630} \leq OD_{control}$, $OD_{control} < OD_{450/630} \leq 2OD_{control}$, $2OD_{control} < OD_{450/630} \leq 4OD_{control}$, $OD_{450/630} > 4OD_{control}$, respectively. An optical density of 0.071 was chosen as guideline to distinguish biofilm producers from those that did not form biofilm.

2.3. Bactericidal Activity of Vinegar Against Streptococcal Biofilm, in vitro

In this study, three different types of vinegar were used on considering that the main components of them were acids specially acetic acid, which has been used as disinfectant against various contaminants (Komiyama *et al*., 2010), furthermore appeared to have antimicrobial potential (Motib, 2012). In addition to that, it is considered cheap and easy to obtain. Besides to vinegar types in our designed study, distilled water (D.W.) was used as a negative control to show inability of biofilm removal in compare to vinegar against study isolates. These types of vinegar include: Date vinegar (DV) (Al-Wafi co., Iraq) with 4.5-5 % acidity; Apple vinegar (AV) (Zer, Turkey) with 5 % acidity; and Grape vinegar (GV) (Al-Wadi Al-Akhdar, Lebanon) with 5 % acidity. All of them considered as table vinegar due to limitation of their acidity not more than 5% in order not to be corrosive for living tissues when used by human.

Determination of bactericidal activity of vinegar against biofilm was performed by using modified microtiter plate method (MMTP) as described previously by Srdjan *et al*. (2000); Tabak *et al*. (2007); Guinta (2010); Sendamangalam (2010) with some modifications. This assay was made by 10µL (1:20) of the raw material of vinegar. Each type was added to prewashed biofilm of standardized bacterial suspension adjusted to 10^5

CFU/ml in microtiter plate for each study isolate, with gentle agitation or vortexing. Then, incubated for 18 hrs. at 37°C. The contents of each well was aspirated. The wells were washed three times with 250 µL of sterile distilled water. Plate was shaken well so that non-adherent

bacteria were removed. The bacteria attached to the walls were then fixed and washed. Furthermore, stained with crystal violet and re-solubilized by the same previous way in quantitative determination of biofilm. The optical density (OD) of each well was measured at the same previous wavelengths by ELISA reader. Similarly the optical density reading interpretations was depended on the previous biofilm criteria.

2.4. Statistical analysis

Data of our designed study were analyzed using the SPSS statistical program (Statistical Package for the Social Science) version 17.0, ANOVA test and LSD test for dependable samples and multiple comparisons. Statistical significance differences were taken with *P*-value <0.01. All the study graphics (bar chart, Pi-chart or other diagrams) were done by using Microsoft Excel **(**Dugard *et al*., 2012).

3. Results

Twenty nine bacterial isolates of *Streptococcus pyogenes* were isolated and identified from infected tonsils that belonged to 19 patients with recurrent tonsillitis. The mean of age groups was 8.21±3.19 years. Those were 12 female (63.2%) and 7 male (36.8%). Out of 29 isolates, 16 (55.2%) bacterial isolates were isolated from tonsil surface, while 13 (44.8%) were isolated from tonsil crypts. The means and Standard deviation for all readings of ODs: 450/630nm were: 0.1842 ± 0.1164, 0.2155 ± 0.1353 for all study isolates (biofilm and non-biofilm producers) which was detected on tonsil surface and crypts, respectively, as shown in table (1)

Regarding the tonsillar biofilm production *in vitro*; out of 29 isolates, 22(75.9%) of *S. pyogenes* were biofilm producers with Mean±StDev (0.2527±0.0866) for Optical densities (ODs) at 450/630nm; whereas 7(24.1%) isolates were negative for biofilm production. Twenty-two biofilm producer isolates were distributed in to 10 (34.5%), 9 (31.0%), and 3 (10.4%) as: Strong producer (SP), Moderate producer (MP), and Weak producer (WP) respectively, as shown in figure (1). Mean±StDev of optical density readings (ODs) at 450/630nm were: 0.3241±0.0580, 0.2197± 0.0304, and 0.1140± 0.0246 for SP, MP, and WP, respectively.

Table 1. Optical density readings at 450/630nm with their means and standard deviations for all *S. pyogenes* isolates (biofilm & non-biofilm producers) isolated from tonsil surface and crypts

Tonsil surface			Tonsil crypts		
no.	Isolate code	ODs: 450/630nm	no.	Isolate code	ODs: 450/630nm
1	1s	0.235	1	1c	0.191
2	2s	0.019	2	2c	0.304
3	3s	0.104	3	3c	0.227
4	4s	0.217	4	5c	0.208
5	5s	0.176	5	6c	0.473
6	6s	0.236	6	9c	0.013
7	8s	0.030	7	11c	0.051
8	9s	0.024	8	12c	0.005
9	10s	0.096	9	20c	0.142
10	11s	0.364	10	21c	0.294
11	12s	0.206	11	22c	0.316
12	20s	0.293	12	24c	0.291
13	21s	0.281	13	25c	0.286
14	23s	0.046			
15	24s	0.285			
16	25s	0.335			
Mean±		0.1842	Mean±		0.2155
StDev		0.1164	StDev		0.1353

The Mean ± StDev of optical density readings (ODs) at 450/630nm for biofilm producer *S. pyogenes* isolated from tonsil surface and crypts were: 2357 ± 0.0830, 0.2732 ± 0.0907, respectively (*P*-value= 0.323).

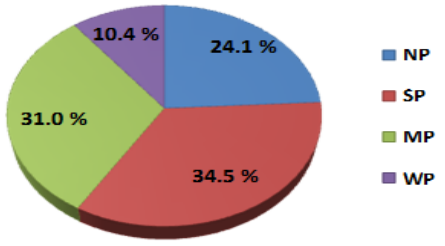

Figure 1. The distribution of *S. pyogenes* isolates according to biofilm production

According to biofilm production criteria, the twenty-two biofilm producer bacteria isolated from surface (54.5%) and crypts (45.5%) were distributed as in figure 2 below:

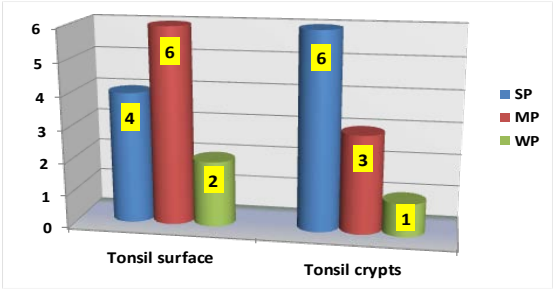

Figure 2. The distribution of biofilm producer *S. pyogenes* numbers isolated from tonsil surface and crypts

The concerned part of the study included examining the impact of three types of vinegar as anti-biofilm *in vitro*, showed that the three types of vinegar, eradicated streptococcal biofilm with excellent degrees. Table 2 demonstrated the readings of optical densities at 450/630nm with their means and standard deviations for

study isolates before and after using of three types of vinegar and distilled water.

Table 2. Optical density readings at 450/630nm with their means and standard deviations before and after using the three types of vinegar and distilled water

no.	Isolate code	ODs: 450/ 630nm	Result of Biofilm production	ODs:450/630nm With different Types of vinegar			Negative control
				Date vinegar	Apple vinegar	Grape vinegar	Distilled water
1	1s	0.235	MP	0.044	0.038	0.046	0.233
2	1c	0.191	MP	0.027	0.052	0.029	0.191
3	2c	0.304	SP	0.061	0.070	0.058	0.303
4	3c	0.227	MP	0.016	0.069	0.040	0.227
5	3s	0.104	WP	0.002	0.004	0.009	0.101
6	4s	0.217	MP	0.018	0.038	0.017	0.215
7	5s	0.176	MP	0.019	0.003	0.011	0.175
8	5c	0.208	MP	0.021	0.001	0.019	0.205
9	6s	0.236	MP	0.023	0.014	0.039	0.233
10	6c	0.473	SP	0.069	0.159	0.183	0.472
11	10s	0.096	WP	0.001	0.013	0.028	0.092
12	11s	0.364	SP	0.033	0.051	0.162	0.364
13	12s	0.206	MP	0.015	0.029	0.029	0.205
14	20c	0.142	WP	0.009	0.002	0.011	0.141
15	20s	0.293	SP	0.051	0.069	0.053	0.292
16	21c	0.294	SP	0.009	0.065	0.068	0.294
17	21s	0.281	MP	0.036	0.070	0.044	0.280
18	22c	0.316	SP	0.011	0.070	0.063	0.315
19	24s	0.285	SP	0.030	0.065	0.037	0.283
20	24c	0.291	SP	0.043	0.065	0.046	0.288
21	25s	0.335	SP	0.021	0.053	0.066	0.333
22	25c	0.286	SP	0.031	0.036	0.027	0.283
Mean±		0.2527		0.02682	0.04709	0.04932	0.2511
StDev		0.0866		0.01811	0.03581	0.04374	0.0870

Our results showed that Date vinegar (DV) prevented biofilm formation in 22(100%) biofilm producer isolates of *S. pyogenes* throughout readings of optical densities and comparison with depending criteria. In Apple vinegar (AV), 21(95.5%) isolates were eradicated, except one. Also, in the third type; Grape vinegar (GV), there was two isolates not eradicated, while 20 (90.9%) isolates were eradicated depending on biofilm criteria. There was a high statistically significant difference before and after using of these three types of vinegar (P-value=0.000<0.01). Otherwise, the negative control (Distilled water) showed no biofilm eradication for all study isolates. There was no significant difference (*P*-value was high 0.931>0.01). On the other hand, there was no statistically significant difference among the three types of vinegar according to *P*-values, as shown in the table 3.

Regarding biofilm categories, the biofilm eradication ability by the three types of vinegar and distilled water was different among biofilm categories (Strong, moderate, and weak) and distributed as shown in figure 3.

Table 3. Multiple comparisons by LSD test, among original readings and the three types of vinegar with control, with Standard errors and *P*-values

		Mean difference	Std. Error	*P*-value
Original	Grape	0.2034091	0.0183829	0.000
	Apple	0.2056364	0.0183829	0.000
	Date	0.2259091	0.0183829	0.000
	Control	0.0015909	0.0183829	0.931
Grape	Original	-0.2034091	0.0183829	0.000
	Apple	0.0022273	0.0183829	0.904
	Date	0.0225000	0.0183829	0.224
	Control	-0.2018182	0.0183829	0.000
Apple	Original	-0.2056364	0.0183829	0.000
	Grape	-0.0022273	0.0183829	0.904
	Date	0.0202727	0.0183829	0.273
	Control	-0.2040455	0.0183829	0.000
Date	Original	-0.2259091	0.0183829	0.000
	Grape	-0.0225000	0.0183829	0.224
	Apple	-0.0202727	0.0183829	0.273
	Control	-0.2243182	0.0183829	0.000
Control	Original	-0.0015909	0.0183829	0.931
	Grape	0.2018182	0.0183829	0.000
	Apple	0.2040455	0.0183829	0.000
	Date	0.2243182	0.0183829	0.000

Regarding biofilm categories, the biofilm eradication ability by the three types of vinegar and distilled water was different among biofilm categories (Strong, moderate, and weak) and distributed as shown in figure 3.

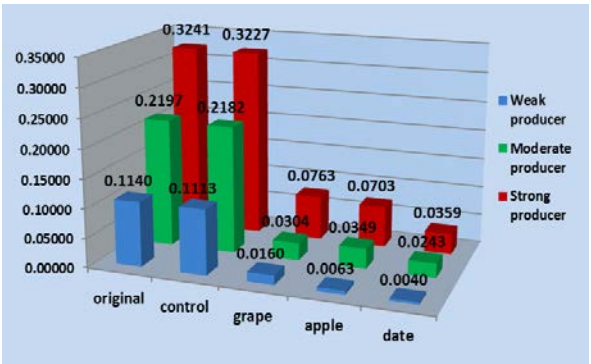

Figure 3. Distribution the biofilm eradication ability means by the three types of vinegar in compared with control and original readings according to biofilm categories

4. Discussion

Recurrent tonsillitis is a common upper aerodigestive tract infection with local and systemic symptoms, such as sore throat, odynophagia, fever, and myalgia. These symptoms can be improved by antibiotic therapy but they tend to relapse (Woo *et al*., 2012). The failure of antibiotic treatment in the eradication of susceptible organisms has recently induced microbiologists to hypothesize the presence of bacteria ordered in communities, attached to surfaces, identified as "biofilms" (Bjarnsholt *et al*., 2011). It has been investigated that *S. pyogenes* was able to form biofilm as an alternative method to escape antibiotic treatment and host defenses leading to recurrent infections (Baldassarri *et al*., 2006). A biofilm is a colony of single or multiple bacterial species embedded in a self-producing polymeric matrix, this matrix guarantees better survival and protection from macrophage action, antibiotics, temperature and pH fluctuations (Galli *et al*., 2007; Bjarnsholt *et al*., 2011; Post *et al*., 2004). One of the best known biofilm-specific properties is antibiotic resistance, which can be up to 1000-fold greater than that seen with planktonic cells (Ogawa *et al*., 2011). So biofilm-associated infections are difficult to eradicate by routine antibiotic doses in compare with planktonic form of bacteria. They need thousands times of doses used for non-biofilm infections (Al-Ani, 2008). As in biofilm formed by *Streptococcus pyogenes* in pharyngitis patients, which evading high antibiotic concentrations greater than 10-folds minimum inhibitory concentration (MIC) for planktonic *S. pyogenes* (Ogawa *et al*., 2011).

Focus on treating established biofilms may need to shift from antibiotic to non-antibiotic therapy to effectively eradicate established biofilms. Among these modalities, were physical (laser-produced pressure waves, pulsed ultrasound) and chemical methods such as using of surfactants, which may soon replace traditional surgical techniques (Smith *et al*., 2011). Researchers have also demonstrated ENT biofilm (ear, nose, and throat) prevention using many various techniques that have been shown to disrupt established biofilms, including the use of probiotics and surfactants (Free *et al*., 2001; Free *et al*., 2003; Rodriguez *et al*., 2004). To the best of our knowledge, this is the first study that has evaluated effect of vinegar on biofilm formed by *S. pyogenes* isolated from recurrent tonsillitis patients.

In this study, with regard to tonsillar biofilm production; 22(75.9%) study isolates of *S. pyogenes* produced biofilm on tonsils, whereas 7 (24.1%) isolates were non-biofilm producer. Ability and inability of biofilm formation by *S. pyogenes* may be due to variance in their strains within group A-streptococci. Different data indicated that there is a necessary protein component for initial adherence and/or aggregation leading to biofilm maturation, made them hypothesized that high levels of streptococcal cysteine protease (SpeB) may be responsible for the biofilm-deficient phenotype, suggesting that SpeB degrades GAS proteins needed for establishment of the biofilm. It is worth nothing that the activation and inactivation of streptococcal regulator of virulence gene (Srv) controlled SpeB production in the strain (Doern *et al*., 2009). Also this result may explain biofilm-related recurrence in tonsillitis patients and presumed the association between frequent tonsillitis and biofilm presence. This result was agreed with Woo *et al*. (2012) that showed presence rate and grade of biofilms were significantly higher in recurrent tonsillitis patients than the control group. Furthermore, other studies reported similar results as mentioned by Chole and Faddis (2003) who identified biofilms in 73.3% of their tonsillitis specimens using light and transmission electron microscopy, whereas Kania *et al*. (2007) reported that biofilms were present in most 70.8% of patients with tonsillitis.

Tonsillar biofilm producing isolates of *S. pyogenes* were distributed into (54.5%) on the surface, and (45.5%) in the crypts; with *P*-value 0.323 as in figure (2), and the means of O Ds were 0.1842 and 0.2155 for all *S. pyogenes* (biofilm and non-biofilm producers) isolated from tonsil surface and crypts, table (1). There was no statistically significant difference between biofilm producer *S. pyogenes* isolated from tonsil surface and crypts but there is a slight preference to the tonsil crypts according to the mean of optical density readings for biofilm production. The tonsil moves dynamically during swallowing and comes into direct contact with food, which makes tonsillar surface vulnerable to biofilm development. In particular, the tonsillar crypts were a preferred site for bacterial attachment and biofilm formation because it is formed by invagination of the surface epithelium and tends to trap foreign material (Balogh and Pantanowitz, 2007; Woo *et al*., 2012). As in Diaz *et al*. (2011) who reported presence of bacterial biofilms in the crypts of 77.28% of the studied patients, he strongly suggested occurrence of a chronic inflammatory underlying pattern with poor immune response.

In our study, biofilm formation ability was evaluated among *S. pyogenes* isolates using standard Microtiter plate assay. These isolates were classified in our study into three groups which are: strong, moderate and weak producer; 10(34.5%), 9(31.0%), and 3(10.4%), respectively, according to previous depending biofilm criteria. Most of isolates were strong and moderate depending on adhesion ability to microtiter plate wells, figure (1). This result is different from biofilm production grading that was reported by Torretta *et al*. (2012) who investigated that (82.4%) were weak producers, and (17.6%) were moderate, but did not report any strong producer isolate among nasopharyngeal biofilm-producing pathogens. Classification of bacteria as moderate, strong or weak biofilm producers regulated by diverse factors, including the growth medium, but still poorly understood (Stepanovic *et al*., 2004). Therefore, they speculate about the reasons for different influence of nutritional content of the growth medium on biofilm formation. One possible explanation for the different response of bacteria to environmental conditions could be the results of mutations in genes that control biofilm formation (Romling *et al*., 1998). While, other results of biofilm assay (tissue culture plate) showed that moderate producer isolates constituted high percentage compared with strong or weak producers. These observations

suggested a strong reliance between growth condition and biofilm formation and using of various sugar supplementations is essential for biofilm formation (Mathur *et al*., 2006).

In the field of studying the effect of vinegar as anti-biofilm, our study presented eradication of Streptococcal biofilm using 4.5-5% date vinegar, 5% apple vinegar and 5% grape vinegar solutions and compared for their removing abilities. Our results explained that all types of used vinegar presented biofilm eradication ability remarkably, *in vitro*. There was no obv ious difference among their prevention ability according to *P*-values as in table (3), also through means of optical density readings, figure (3). The main constituents of vinegar represented by acids specially acetic acid (Jafari *et al*., 2012), so vinegar used as anti-biofilm in our designed study, due to antibacterial effect of acetic acid that treats infections caused by bacteria or fungus (Jaber *et al*., 2011). Furthermore, vinegar used as a surfactant against *S. pyogenes* (Motib, 2012). Acetic acid-based solutions (vinegar) used for the disinfection and treatment of oral inflammatory processes (as a mouthwash) and as an anti-septic for sores which has been reported in previous studies (Utyama, 2003).

Although there was no o bvious difference among types of vinegar, Date vinegar was the best for biofilm prevention, which is possibly due to one of its components that have antibacterial activity besides acetic acid. Al-daihan and Bhat (2012) showed that Date extracts were effective inhibitors for bacterial growth. This may be due to presences of carbohydrates, alkaloids, steroids, saponins, flavonoids and tannins in this fruit according to the chemical analysis. They recorded mean of inhibition zones as 18 m m ± 0.08 for these extracts against *S. pyogenes*. Also, apple vinegar showed amazing biofilm eradication ability however less than date vinegar. This antimicrobial effect may be due to high content of phenolic compounds in apple as proved by Alberto *et al*. (2006) who demonstrated direct relationship between phenolic content of apple extract and the antimicrobial effect on h uman pathogens. Sendamangalam, (2012) explained that polyphenols reduced streptococcal biofilm formation through inhibition of exopolymers-producing enzymes, exopolymers are a major component in biofilm formation. He observed that biofilm reduction ability was ranged between 62.8 - 75.7% for used polyphenols. Grape vinegar had the least effect in compared with other two types, however it prevented biofilm remarkably. This may be due to difference in concentrations of grape components, as explained by Smullen *et al*. (2006) who concluded that grape extract containing high levels of polyphenols inhibited streptococcal biofilm. It has been found that grape extracts include polyphenols, flavanol, catechins and anthocyanins, which exhibited different antimicrobial activity according to their concentrations.

The study concluded that there was obvious correlation between biofilm production by *S. pyogenes* and recurrent tonsillitis, while there was no significant difference in biofilm production between *S. pyogenes* isolated from tonsil surface and crypts. Furthermore, our study concluded that the three types of vinegar eradicated streptococcal biofilm remarkably, *in vitro*, although date vinegar was the best for eradication of streptococcal biofilm.

On the other hand, our results showed a huge difference in the biofilm eradication ability before and after using of vinegar types, but there was no effect for distilled water (negative control), figure (3). These results correspond with Jafari *et al*. (2011) who found that 99-100% of biofilm formed on the denture surface was removed by using two concentrations of white vinegar (5,10)% and 1% sodium hypochlorite.

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Effects of COX-1 and COX-2 Inhibitors in L- Nitro-L-Arginine Methyl Ester Induced Hypertensive Rats

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Abstract

Administration of L-arginine analogues, such as N-nitro-L-arginine methyl ester (L-NAME) is related with cardiovascular and renal functional alteration. The present experiment was planned to study the possible hemodynamic, renal and liver effects of cyclooxygenase (COX)-1(Aspirin) and COX-2 (Celecoxib) inhibitors in L-NAME induce hypertensive rats. The experimental rats were divided into four groups, each with six animals and the treatments were continued for 2 weeks as the following: Group 1: Control. The rats were given standard rat chow and tap water ad libitum. Group 2: L-NAME. The rats were given standard rat chow and L-NAME (20mg/kg body weight). Group 3: COX-1 inhibitor. The rats were supplied with standard rat chow with aspirin (1000 mg /kg diet) and L-NAME Group 4: COX-2 inhibitor . The rats were supplied with standard rat chow with celecoxib (1000 mg /kg diet) and L-NAME. Results showed that the systolic blood pressure (SBP) was elevated in control rats in comparison with L-NAME group. In the group of receiving COX-1 inhibitor, SBP significantly reduced, while COX-2 inhibitor reduced it but not significantly. Heart rate (H.R) was also changed after COX-2 inhibitor administration ,while COX-1 inhibitor did not change it significantly. COX-1 administration increased serum Na^+ levels ,while serum K^+ levels was significantly increased in COX-2 group rats compared with the L-NAME group. Supplementation of L-NAME for 15 days produced a significant increase in serum aspartate transaminase (AST) activity compared with the control group. Statistical analysis revealed that a significant reduction in alanine transaminase (ALT) activity was observed by COX-2 administration compared with the L-NAME. COX-2 inhibitor markedly elevated serum creatinine level compared with the L-NAME group. In conclusions, the results suggested that aspirin rather than celecoxib reduces SBP and in contrast to aspirin, celecoxib alter kidney functions through elevation of serum creatinine level, but it may attenuate liver functions through reduction of elevated serum ALT activity by L-NAME administration.

Keywords: COX , Aspirin , Celecoxib, N-Nitro-L-Arginine Methyl Ester

1. Introduction

Administration of L-arginine analogues, such as N-nitro-L-arginine methyl ester (L-NAME) are related with cardiovascular and renal functional alteration (Kunes *et al*., 2004). The mechanisms by which L-NAME produce hypertension may be due to vasoconstrictor effect of angiotensin II (Ag II) which in turn removes the normal modulatory influence of nitric oxide (NO). Furthermore, previous researchers (Navarrow *et al.,* 1994; Zanchi *et al.,* 1995) found that long-term inhibition of NO synthase may due to increase in plasma epinephrine and norepinephrine, consequently they increase blood pressure (BP). Some of the experimental models showed that hypertension produced by NO synthesis inhibitor is association withincreased oxidative stress (Sainz *et al*., 2005).

The prostaglandins perform a homeostatic role within the normal and compromised kidney (Imig,2000) Two isoforms of COX have been identified: COX1 and COX2. COX1 is constitutively expressed in most tissues examined and is thought to be involved in maintaining basic cellular function. In contrast, COX2 is induced by physiological and pathophysiological stressors and plays important roles in the cellular response to stress (Herschman,1996 and Wadleigh *et al.,* 2000). Non steroidal anti-inflammatory drugs (NSAIDs) such as aspirin inhibit the COX enzymes, reducing inflammation. COX-1 is the constitutive isoform (Vane *et al.,* 1998), whereas COX-2 is the inducible isoform and considered to be a mediator of inflammatory disease (Venturini *et al*., 1998). In addition to their role in inflammation, prostaglandins are important regulators of vascular tone, salt and water balance, and renin release, and nonselective NSAID exhibit adverse effects, including salt retention

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and reduced GFR, which may elevate BP or make pre-existing hypertension worse (Johnson, 1997).

Medullary prostaglandin E2 (PGE2) plays an important role in regulating NaCl and water reabsorption in the medullary thick ascending limb and collecting duct (Stokes, 1981). Because COX-1 is abundantly and constitutively expressed in both cortical and medullary collecting duct, COX-1–derived prostanoids have been hypothesized to be involved in the natriuretic response, and in this regard, acutely increasing renal interstitial hydro- static pressure by direct renal interstitial volume expansion will induce increased sodium excretion, which is blunted by infusion of nonselective NSAID but not COX-2 inhibitors (Gross,1999).

Furthermore, in a rat model of cirrhosis and ascites, a COX-1 selective inhibitor but not a COX-2 selective inhibitor decreased sodium excretion and impaired the diuretic and natriuretic responses to furosemide (Lopez-Parra *et al.,* 2002). Recently, administration of selective COX-2 inhibitors or COX-2 gene knockout has been shown to accentuate the vasoconstrictor effects of angiotensin II in mice (Qi, 2002). Deterioration of renal function may also be observed in patients receiving non steroidal anti-inflammatory drugs (NSAIDs) or COX2 selective inhibitors (Whelton,1999).

However, for the first time Folia (2005) observed that antihypertensive treatment which advantageously decreased the agreeability of erythrocytes was diminished by a high dose of aspirin (300 mg/day).

Results for celecoxib are somewhat different. One study found that cardiovascular side effects, such as myocardial infarction, stroke, or heart failure, were associated with prolonged treatment with celecoxib (Solomon *et al*., 2005). In addition, Gupta *et al.* (2007) concluded that chronic administration of celecoxib may have a d amaging effect on kidney, as evident through altered histopathology and renal functions. This damage may be mediated by oxidative stress. COX-2 is widely expressed throughout the kidney, and inhibition of this enzyme is contributory to reduced glomerular filtration, salt and water retention, and blood pressure elevation (Krum et al., 2006). Thus, the aim of the current study was to investigate the hemodynamic and renal effects of COX -1 and COX-2 inhibitors in L-NAME induced hypertensive rats.

2. Materials and Methods

2.1. Animals and Housing

Twenty four adult female albino rats were used in this study. Female rats were used, so few studied are done on females because they have resistance in some models of hypertension rather than L-NAME-induced hypertension. All rats were weighing about (240 - 280 gm) and (7-9) weeks of age at the time when the experiment was started. Animals were housed in plastic cages bedded with wooden chips. They were housed under standard laboratory conditions, 12:12 light/dark photoperiod at 22 ± 2 ºC. The animals were given standard rat pellets and tap water ad libitum.

2.2. Experimental Design

The present experiment was planned to study the possible hemodynamic, renal and liver effects of COX-1 and COX-2 inhibitors in L-NAME induce hypertensive rats. The experimental rats were divided into four groups, each with six animals and the treatments were continued for 2 weeks as follows:

Group 1: Control. The rats were given standard rat chow and tap water *ad libitum*.

Group 2: L-NAME**.** The rats were given standard rat chow and L-NAME at dose (20mg/kg body weight).

Group 3: COX-1 inhibitor (Aspirin). The rats were supplied with standard rat chow with aspirin (1000 mg /kg diet) and L-NAME at dose (20mg/kg body weight).

Group 4: COX-2 inhibitor (Celecoxib). The rats were supplied with standard rat chow with celecoxib (1000 mg /kg diet and L-NAME at dose (20mg/kg body weight).

2.3. Blood Pressure and Heart Rate Measurements

Measurements of SBP and heart rate were obtained at the end of the experiment by the tail-cuff method in all groups using power Lab (AD Instruments, power lab 2/25). During the week before treatment the rats were trained to become accustomed to the blood pressure measurements. Rats were placed in a restraining chamber and warmed to an ambient temperature of approximately 37C^{o}, typically taking about 10-15 minute after that occluding cuffs and pneumatic pulse transducers were placed on the rats' tails. Six readings were taken for each rat, the highest, lowest and any associated with excess noise or animal movement were discarded. The average was taken of the remaining readings to generate a v alue for a given rat for that week.

2.4. Biochemical Determination Determination of Serum AST and ALT Activity

Serum AST and ALT activities were determined by colorimetric method kit ((Biolabo . SA, France)

2.5. Determination Of Serum Creatinine Level

Creatinine level was determined by colorimetric method kit (Biolabo . SA, France). Creatinine in alkaline picric acid solution, forms a color complex in which the absorbance was measured at 490 nm using spectrophotometer

2.6. Determination of Serum Uric Acid Level

Serum uric acid level was determined by uricase method, using colorimetric test kit (Biolab, France)

2.7. Determination of Serum Total Protein Level

Serum total protein levelwas determined by biuret method, using colorimetric testkit (Biolab, France)

2.8. Determination Of Serum Sodium And Potassium Ion Concentrations

Serum Na+ and K+ ion concentrations were determined by using flame photometer (Jenway Flame Analyzer, USA)

2.9. Serum Total Calcium Ion Determination

Ca^{2+}-Kit enables colorimetric determination of total calcium(Chawla, 2003), without deproteinization. In serum, the calcium kit reacts with methylthymol blue

indicator (MTB) in an alkaline medium. The color intensity of the Ca^{2+}-MTB complex, measured at 612 nm, is proportional to the quantity of calcium present in the sample. The kit was obtained f rom (BIOLABO.SA, France).

2.10. Statistical Analysis

All data were expressed as means ± standard error (SE) and statistical analysis was carried out using available statistical soft ware (SPSS version 15). Data analysis was made using one-way analysis of variance (ANOVA). The comparisons among groups were done using Duncan post hoc analysis. P values <0.05 were considered as significant.

3. Results

Figure 1 s how the changes in SBP and H.R in L-NAME group rats. As expected, the SBP was elevated from 112.6± 2.752 mm Hg in control rats to 166.8± 2.561 mm Hg in L-NAME group . H.R was also raised from 353.6 ± 5.420 bpm to 397.6± 15.33 bpm .In the group of receiving COX-1 inhibitor, SBP significantly reduced to 144.8± 6.335 mm Hg ,while COX-2 inhibitor reduced it but not significantly. H.R was also changed after COX-2 inhibitor administration ,while COX-1 inhibitor did not change it s ignificantly (Figure 2). Cox-1 administration increased serum Na^+ levels ,while serum K^+ levels was significantly increased in COX-2 group rats(6.829 ±0.2892 mmole/L) compared with the L-NAME group (5.619 ±0.2511 mmole/L).Neither COX-1 nor COX-2 administration caused change in serum Ca^{2+} shown in Table 1.

Supplementation with 30 mg/100ml drinking water of L-NAME for 15 days produced a significant increase in serum AST (86.64± 2.678 IU/L) compared with the control group(68.67± 4.3201 IU/L). AST (IU/L) did not change by COX-1 and COX-2 supplementation.(Table 2).Statistical analysis revealed that a significant reduction in ALT(IU/L) was observed by COX-2 administration (25.82± 3.392) compared with the L-NAME group (38.77±1.3882). No significant change in serum ALT was obtained by in COX-1 group rats. The reduction of serum ALP (IU/L) that was detected by L-NAME administration was significantly returned to the control group by COX-2 inhibitor, while COX-1 inhibitor did not change it significantly.

As illustrated in figure 1,COX-2 inhibitor markedly elevated serum creatinine (0.942 ±0.061 mg/dL) compared with the L-NAME group (0.679±0.027 mg/dL).On the other hand, serum uric acid (mg/dL) was statistically increased in L-NAME group (1.9235±0.362).There were no statistical difference in serum total protein among the studied groups.

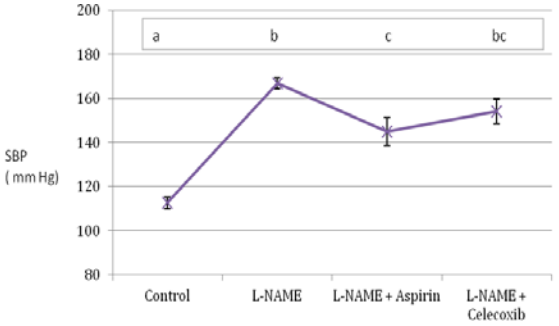

Figure 1. Effects of aspirin and celecoxib on SBP

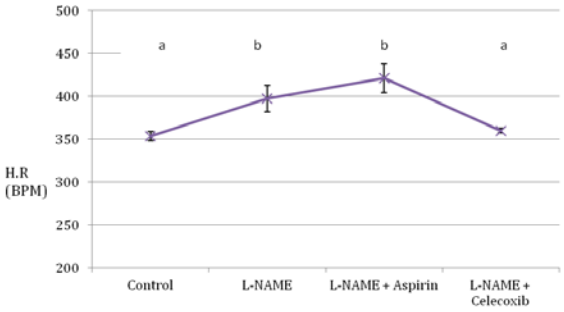

Figure 2. Effects of aspirin and celecoxib on serum H.R

Table 1. Effects of aspirin and celecoxib on serum Na^+, K^+ and total Ca^{2+}

Parameters / Treatments	Control	L-NAME	L-NAME + Aspirin	L-NAME + Celecoxib
Serum Na^+ (mmol/L)	154.4 ± 2.673^{b}	146.9 ± 1.984^{ab}	157.2± 4.3054^{b}	137.3± 5.5614^{a}
Serum K^+ (mmol/L)	6.059± 0.3508^{ab}	5.619 ± 0.2511^{a}	6.299± 0.490^{ab}	6.829 ± 0.2892^{b}
Serum total Ca^{2+}(mg/dl)	6.2888± 0.378^{a}	6.6156± 0.442^{a}	5.6804± 0.161^{a}	6.7747± 0.339^{a}

The data presented as mean ± SE measured after 2 weeks of the treatments in all groups (Control , L-NAME(L-nitro-arginine methyl ester) , L-NAME + Aspirin (COX-1 inhibitor), , L-NAME + Celecoxib (COX-2 inhibitor).

The same letters (a, a, a) mean non significant differences while the different letters (a, b, c) mean significant differences at $p<0.05$

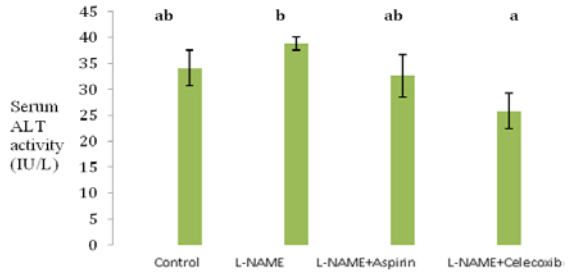

Figure 3. Effects of aspirin and celecoxib on serum ALT activity

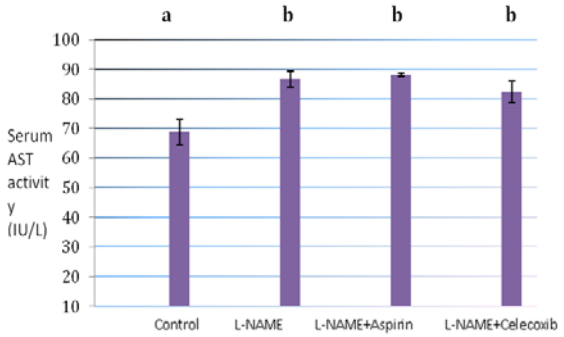

Figure 4. Effects of aspirin and celecoxib on serum AST activity

Table 2. Effects of aspirin and celecoxib on serum creatinine , uric acid and total protein levels

Parameters / Treatments	Control	L-NAME	L-NAME + Aspirin	L-NAME + Celecoxib
Serum creatinine (mg/dL)	0.554± 0.0342[a]	0.679± 0.027[ab]	0.841± 0.1374[bc]	0.942 ± 0.061[c]
Serum uric acid (mg/dL)	1.117± 0.110[a]	1.9235± 0.362[b]	1.1535± 0.091[a]	1.4517 ± 0.231[ab]
Serum total protein (gm/dL)	5.741 ± 0.4486[a]	6.388± 0.314[ab]	7.4917± 0.625[b]	6.1750 ± 0.325[ab]

The data presented as mean ± SE measured after 2 weeks of the treatments in all groups (Control , L-NAME(L-nitro-arginine methyl ester) , L-NAME + Aspirin (COX-1 inhibitor), , L-NAME + Celecoxib (COX-2 inhibitor).The same letters(a ,a ,a) mean non significant differences while the different letters(a, b, c) mean significant differences at $p<0.05$

4. Discussion

In the present study, results show that SBP was elevated by L-NAME for 2 weeks of administration at a dose of 30 m g/100ml in drinking water. As it was mentioned previously, L-NAME produce hypertension due to its elevation of ANGII (Navar *et al*., 1996). L-NAME basically inhibit COS synthase activity and reduce the level of NO (Oktem *et al.,* 2011). NO is a potent vasodilator (Vallance and Chan, 2000). Reduction of NO levels increases blood pressure due to overriding vasoconstrictors, or L-NAME increases epinephrine levels (Zanchi *et al*., 1995), or due to an increase in oxidative stress (Saniz *et al*., 2005).The precise mechanism by which oxidative stress increases blood pressure in not fully understood. One possible mechanism is that oxidative stress may increase some of the vasoconstrictors like endothelin-1 (ET-1), (Wedgwood and Black ,2005).ET-1 is a potent vasoconstrictor (Methew *et al*., 1997) ,thereby causes hypertension. On the other hand other investigators resulted that NO deficiency causes accumulation of superoxide anion or hydrogen peroxide which may have prohypertesive actions (Shokoji *et al*., 2003). ET-1 also increases H.R as we obtained from the current results shown in Table 1.

Statistical analysis revealed that COX-1 inhibitor caused a significant reduction in SBP, but it could not returned to the control group. However, COX-2 reduced SBP but the degree of reduction did not reach the significant values. The possible mechanism of COX-1 effects may be due to reducing some of the prohypertensive effects of prostaglandins especially prostaglandin F2 and Thromboxane A2 (Kim *et al*., 2010). On the other hand, acetylsalcylic acid reduce vascular $\cdot O2^-$ production through lowering nicotinamide diphosphate hydrogen oxidase activity (Rong *et al*., 2002). As previously mentioned , free radicals increases blood pressure(Shokoji *et al*., 2003). Natriuretic effects of COX-1 inhibitor also may be different factors to reduce SBP. Furthermore, recently, Zhang *et al.* (2007) observed that aspirin reverses adrenocorticotropic (ACTH) hormone. ACTH releases cortisol, and the water and salt retention increases consequently blood pressure will raises. However, COX-2 inhibitor reduced heart rate significantly, but such reduction did not affect systolic blood pressure and the effects of COX-2 on systolic blood pressure in the current results is consistent with Richter *et al*. (2004) ,in their experiment in renovascular hypertensive rats observed that blood pressure was not affected by COX-2 administration. In the recent study, Zaitone *et al.* (2011) observed that COX-2 did not affect blood pressure. However, the exact mechanism by which COX-1 and COX-2 affect hemodynamics remain to be explained.

As shown from the present results, serum Na^+ was slightly increased by COX-1. On the other hand, COX-2 markedly elevated serum K^+ level compared with the L-NAME group rats. The same results regarding COX-1 related with serum Na^+ regulation was obtained by Lpez-Parra *et al*. (2002), they showed that COX-1 inhibitor but not a COX-2 selective inhibitors decreased sodium excretion and impaired the diuretic and natriuretic response to furosemide. It is well known that reduction of Na^+ excretion will elevate its values in the serum. However, our result (Effects of COX-2 on serum Na+ level) in this point is in contrast to Richter *et al*. (2004),they concluded that celecoxib treatment did not alter serum sodium in two kidney-one clip renovascular hypertensive rats.

The current results also revealed that COX-2 inhibitors significantly elevated serum K^+ level. One possible mechanism is that COX-2 inhibitors which may have a damaging effect on k idneys (Gupta *et al.,* 2007) may reduce K^+ secretion, as a result serum K^+ levels increases. According to the present results regarding effects of COX-1 and COX-2 inhibitors on serum Ca^{2+} level , both of these enzyme inhibitors may have not associate with parathyroid hormone, calcitonin and vitamin D metabolism, otherwise serum C2+ in contrast to the obtained results be reduced or increased compared with control group.

The enzymatic activity of AST and ALT were studied to estimate liver and heart malfunctions. As shown in figure (4), serum AST activity was significantly increased after L-NAME administration. Previous researchers observed that serum AST activity increased after myocardial infarction and hepatic parenchymal injuries (Burtis and Ashwood, 1994). Serum ALT activity was also elevated in group of L-NAME. Sainz *et al.* (2005) showed that hypertension produced by NOS inhibitor is significantly related with oxidative stress. Free radicals

included cell damage may be determined by MDA concentration (Oktem *et al*., 2011). Deniz *et al*. (2006) observed that NOS inhibitor induced hypertension increases malonaldehyde concentration. Other possible causes of AST and ALT activities elevation by L-NAME may be related with an increase of some vasoconstrictors such as ANG II and ET-1 ,which may thy enhance free radical formation (Schnackenberg, *et al*., 2000; Touyz, 2003).

Neither COX-1 nor COX-2 inhibitors ameliorate serum AST activity, whereas serum ALT activity slightly reduced by COX-1 and significantly ($P< 0.05$) reduced by COX-2 inhibitor. Renna *et al.* (2009) concluded that chronic aspirin treatment normalized relative heart weight and vascular remodeling. Furthermore, Washino *et al.* (2010) according to their results suggested that celecoxib effectively ameliorates the necritic action and the oxidative stress induced by tetrachloroacetic acid CCL4. Ozturk *et al.* (2006) also confirmed the beneficial effect of celecoxib in hepatic ischemias reperfusion injury and they suggested that celecoxib may protect the liver. On the other hand, Malek and Saleh (2009) resulted that celecoxib produce significant reduction in serum ALT activity, MDA and alpha tumor necrotic factor.

Results of the present study shows that COX-2 inhibitor markedly increased serum creatinine compared with the L-NAME group. Gupta *et al.* (2007) obtained the same result showing chronic administration of celecoxib alter renal functions .In another study, patient receiving COX-2 selective inhibitors, deterioration of renal function may be observed (Whelton, 1999). However, this elevation of creatinine level by COX-2 in our study is in contrast to Richter *et al.* (2004), they resulted that celecoxib did not alter GFR and serum creatinine. The present results also showed that L-NAME administration significantly raised serum uric acid. Reduction of NO levels elevate Ang II and ET-1 which represent key mediators for kidney damage (Amann *et al*., 2001; Dhaun *et al*., 2006), consequently serum uric acid may from the blood (Kumar *et al.*,2000). In conclusions, the results suggested that aspirin rather than celecoxib reduces SBP and in contrast to aspirin ,celecoxib alter kidney functions through elevation of serum creatinine level, but it may attenuate liver functions through reduction of elevated serum ALT activity by L-NAME administration.

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Is Gaza Sandy Shoreline Region Contaminated with Human Gastrointestinal Parasites?

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Abstract

The study was implemented to test if the sandy shoreline of Gaza city is contaminated with human gastrointestinal parasites or not and to determine the types of intestinal parasites and the extent of contamination. A total of 104 sand samples (52 dry sand and 52 wet sand) were analyzed during the summer season period. Samples were collected from the study area of about 12km along the seashore region of Gaza City. Dry samples and wet sand samples were analyzed using water-sedimentation technique and a light microscope. The results showed that the percentage of the parasitic contamination was 40.4% of the wet sand samples and 34.6% of the dry sand samples along the shoreline region of the Gaza City. The human gastrointestinal parasites detected were the following: *Ascaris lumbricoides, S. stercoralis, E. vermicularis, E. histolytica/dispar*, *G. lamblia, E. coli* and *Taenia. spp*. The findings showed that there was no statistically significant difference in the concentration of parasitic contamination between the dry and wet sand samples at confidence level of p-value < 0.05. It is recommended to conduct a periodical routine sampling of sand at the swash zone because the results of this study showed that the wet and the dry sand may pose a high level of health risk. Residents should be informed clearly by posting signs indicating polluted areas to keep them safe.

Keywords: Sandy shoreline**,** Intestinal parasites**,** Contamination, Wastewater, Gaza city

1. Introduction

The population of Gaza City is about 552,000 (PCBS, 2011), and receives a water supply through the Coastal Municipalities Water Utility (CMWU) water supply system. High percentage of the wastewater that is generated in Gaza City is currently discharged without sufficient treatment into the sea in addition to the Wadi Gaza's wetland effluent channel for the raw sewage from refugee camps adjacent to the watercourse, estimated of about 6-8MLD (Hilles and Abu Amr, 2010).

The population of Gaza Strip continues to grow rapidly, which increase the amounts of poorly treated or untreated sewage being discharged into the coastal water. With the Palestinian population growth rate of around 4.8% per annum, which would result in a doubling of the population in 15 years, effective management and sustainable development of Gaza resources will be a huge challenge for the Palestinian Authority (UNEP, 2003).

In Gaza City, there is only one insufficient and inefficient wastewater treatment plant (GWWTP) which is considered to be the largest in Gaza Strip. Insufficient means that the quantity of the wastewater discharged from the city and arrived to the plant exceeds its capacity, while, inefficient means that the plant suffers from lack of maintenance and operational problems. GWWTP discharges about 50MLD of partially treated wastewater directly into the sea along with 10MLD of untreated wastewater (raw sewage) is currently discharged directly into the sea of Gaza City (EWASH, 2009).

The only study conducted in Gaza was the assessment of total coliform and faecal coliform in Gaza sea shore (Elmanama, 2004). The present study is the first one to assess the parasitic contamination of Gaza sea shore.

The lack of sufficient wastewater treatment facilities makes wastewater which discharges into the sea the main source of pollution of the coastal zone of Gaza Strip. There are more than 20 individual sewage drains, ending either on the beach or a short distance away in the surf zone. Insufficient number of sewage treatment plants in operation, combined with poor operating conditions of available treatment plants, and the present disposal practices are likely to have an adverse effect on the quality of seawater (EQA and UNEP, 2005).

The main aim of the current study is to examine if Gaza shoreline region is contaminated with human gastrointestinal parasites (identify them up to the species

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level) and to determine the extent of parasitic pollution in the seashore sand..

2. Materials and Methods

2.1. The study area:

About 12km of Gaza City shoreline was divided into six sampling zones in order to facilitate the sampling process as it is shown in table 1 and Fig 1.

2.2. Sampling processes

Sampling were conducted throughout the summer season months (from June to October, 2011).During the study 500gm of sand were collected from every sample of the previously mentioned areas (Table 1 and Figure 1) of 2m², which were divided into five cores of 100gm taken from each corner of the sampling site and another one from the central part of the area within the superficial layer of the ground at an approximate depth of 5-10cm (Colli *et al*., 2010). The samples were stored in suitable plastic bags, and directly labeled and signed with special water-resistant pen. Dry sand samples were collected from non flooded areas (above high tide line) in the areas out of reach of seawater, and wet sand sample was taken from an intermediate area between the dry sand area and the seawater (Swash zone or Intermediate zone) where it was very close to the seashore and the seawater usually reach and moisten the sand in these sites, and where bathers could be found most of the time.

Table 1. Zones of Sampling and Related Information

Zone Symbol	Zone Boundaries	Zone length	Total number of samples	Number of samples	
				Wet Sand	Dry Sand
A	From Wadi Gaza to Al-Zahra City	1800m	**(24 Samples)** -First 5 samples every50m. -Second 5 samples every 250m.	12	12
B	Al-Zahra City to Al-Baydar resturant	2000m	**(10 samples)** -Every 500m	5	5
C	From Al-Byder restaurant to Khalel Alwazer mosque	1800m	**(12 samples)** - Sample every 300m.	6	6
D	From Khalel Alwazer mosque to the southern part of the Gaza marina	2500m	**(24 samples)** - Sample every 200m.	12	12
E	The basin of the Gaza marina	450m	**(8 samples)** -Every 100m.	4	4
F	From the northern part of the Gaza marina to the Intelligence Building	2800m	**(26 samples)** - Sample every 200	13	13
Total	**Study area**	**12km**	**104 samples**	**52**	**52**

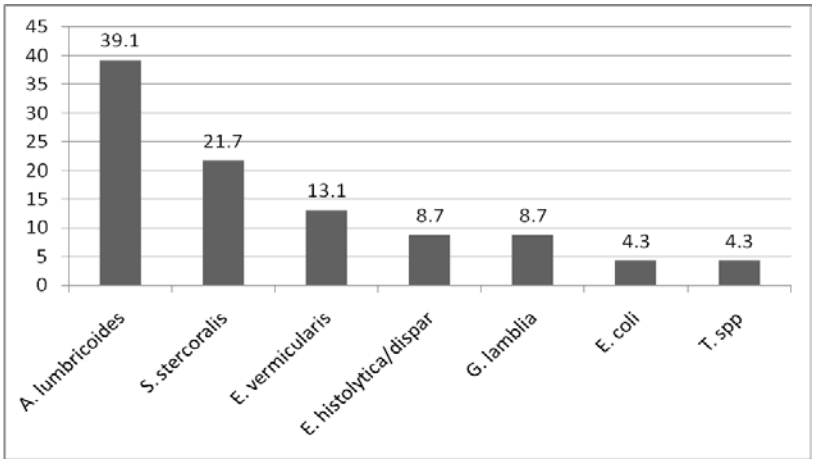


Figure 1. Distribution of the detected parasites in the dry sand within the six zones

2.3. Using water-sedimentation technique,

35gm of sand was diluted (washing gently for 15 sec) in 150 ml of distilled water, filtered through a sieve meshes (75µm) and allowed to settle for between six and eight hours in a suitable 250 ml measuring cylinder. Two ml of the surface of the cylinder (floated parasites in the upper part of the aqueous solution) were taken and discarding the rest of the supernatant, about eight ml of the stagnant sediment were collected, and the two amounts were centrifuged together to concentrate the sample by 1500 rpm for about 10 min and a concentrated sediment was collected (Colli et al., 2010).

The sand analysis process was completed by applying the following steps: One drop of the sediment was placed in the center of the slide. The drop was covered with a cover slip by holding the coverslip at an angle, touching the edge of the drop, and gently lowering the coverslip on top of the slide so that air bubbles are not produced. The slide was examined with 10X objective or, when needed for more identification, higher power objectives of the microscope have been applied in a systematic manner (either up and down or laterally) so that the entire coverslip area was surveyed. When organisms or suspicious objects were seen, switching to higher magnification was necessary to see more detailed morphology of the object in question. The sediment were stored in a labeled suitable tube which is known as opened rove (with a sharp bottom and snap cap) for further analyses and inspection.

3. Results

The present study showed a diversity of human gastrointestinal parasites in both dry and wet sand in Gaza sandy shoreline region.

The results of the dry and wet sand analysis for the entire study area (six zones = 52 samples) are shown in Table 2. Eighteen (34.6%) were found to be contaminated with human gastrointestinal parasites. It was found that 14 samples were contaminated by one type of parasites (single) and 4 samples were contaminated by several types of parasites (mixed).

For wet samples, 21 samples (40.4%) were contaminated by human gastrointestinal parasites: 18 samples were contaminated by one type of parasites (single) and 3 samples were contaminated by more than one species (mixed) of human gastrointestinal parasites as shown in Table 2.

Figure 1 shows that the dry sand samples were contaminated with seven species of human gastrointestinal parasites, distributed in order as follows: 39.1% *Ascaris lumbricoides,* 21.7% *S. stercoralis,* 13.1% *E. vermicularis,* 8.7% *E. histolytica/dispar*, 8.7 % *G. lamblia,* 4.3 % *E. coli* and 4.3 % *Taenia.* spp.

According to Figure 2, the results of contamination with parasites in dry sand according to the different six zones shows that zone (A) has the highest level of contamination with 44.4% of polluted samples, followed by 22.2% in zone (D), 22.2% in zone (B), 5.6% in zone (C), and finally 5.6% in zone (E). No parasitic pollution was detected in zone (F).

Figure 3 shows that wet sand samples were contaminated with six species of human gastrointestinal parasites and distributed as follows: 58.2% *S. stercoralis,* 25.0% *A. lumbricoides* and 4.2% for each of *E. histolytica*/*dispar*, *G. lamblia, E. coli* and *Taenia.* spp.

As illustrated in Figure 4, contamination with parasites in wet sand depending on the different six zones shows that zones (A) and (D) have the highest level of contamination with 28.6% for each zone, followed by 19.0% in zone (C), 14.3% in zone (B) and finally by 9.5% in zone (E). No parasitic pollution was found in zone (F).

The images of the human gastrointestinal parasites which have been recorded and identified in the samples from the six zones and all sampling sites are presented in Figure 5.

No statistical significant difference in the concentration of parasitic contamination between the dry and wet samples at a confidence level of a p-value < 0.05 was found (Table 3.).

Table 3 illustrates the results of a single factor one way-ANOVA test for the spatial variation in the parasitic contamination within the six different zones along the entire study area to examine whether there is a significant statistical difference in the contamination level through those zones and the level of significance. The results indicate that there is a significant variation among the zones within the confidence level of a p-value of < 0.05).

Table 3 shows a significant mean difference between the higher polluted zone (A) and the other zones. Also, there was a significant mean difference between uncontaminated zone (F) compared to the other zones using multiple comparisons (LSD) as a statistical analysis method.

Table 2. Percentage of Contaminated Dry and Wet Sand Samples

		Dry Sand		Wet Sand	
		No.	(%)	No.	(%)
Contaminated samples	Single	14		18	
	Mixed	4		3	
	Total contaminated samples	18	34.6	21	40.4
Uncontaminated		34	65.4	31	59.6
Total samples		52	100.0	52	100.0

Table 3. Statistical analysis done during the study

t-Test for the Means of the Dry and Wet Sand	Independent Samples Test				
	Levene's Test for Equality of Variances		t-test for Equality of Means		
	F	Sig.	t	df	Sig. (2-tailed)
Equal variances assumed	1.399	0.240	0.603	102	0.548
Equal variances not assumed			0.603	101.903	0.548

2. One way –ANOVA Test for the Parasitic Pollution within the Six Zones (A, B, C, D, E, F)

	Sum of Squares	df	Mean Square	F	p-value
Between Groups	11.133	5	2.227	13.011	.001
Within Groups	43.467	254	.171		
Total	54.600	259			

3. Multiple Comparisons (LSD) for the Parasitic Pollution Within the Six Zones

(I) ZONE	(J) ZONE	Mean Difference (I-J)	Std. Error	p-value
A	B	.2300(*)	.09848	0.020
	C	-.1397	.09356	0.137
	D	-.1057	.07522	0.161
	E	-.2500(*)	.10681	0.020
	F	-.4500(*)	.07406	0.001
F	A	.4500(*)	.07406	0.001
	B	.6800(*)	.09736	0.001
	C	.3103(*)	.09238	0.001
	D	.3443(*)	.07374	0.001
	E	.2000	.10578	0.060

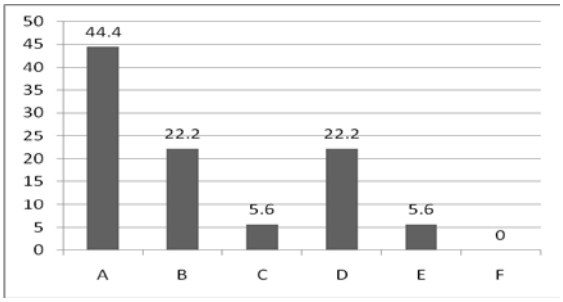


Figure 2. Percentages of contamination in dry sand according to the six zones

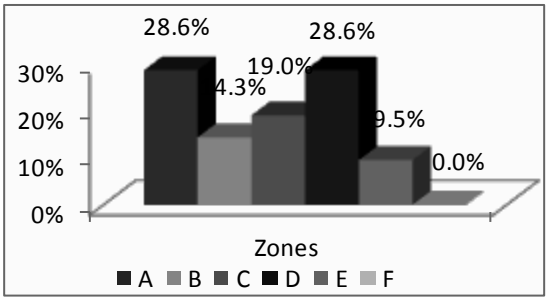


Figure 4. Percentages of contamination in the wet sand according to the six zones

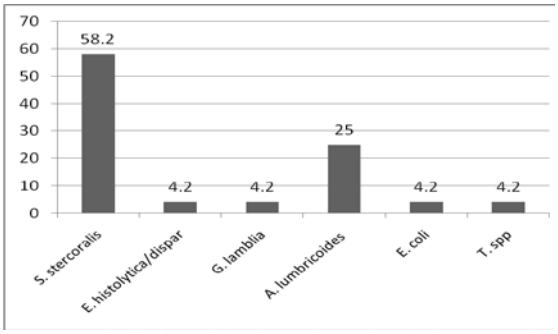
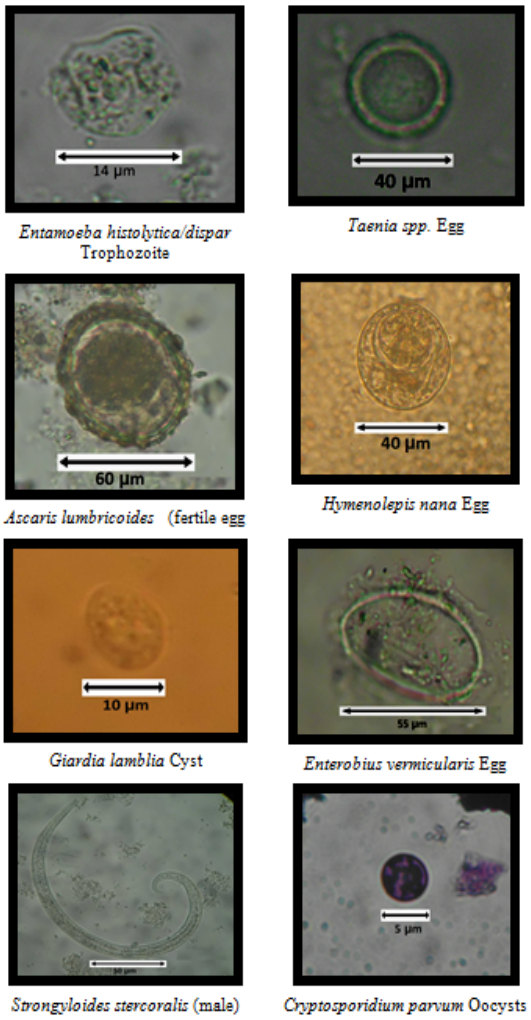


Figure 3. Distribution of the detected parasites in the wet sand within the six zones



Entamoeba histolytica/dispar Trophozoite | *Taenia spp.* Egg

Ascaris lumbricoides (fertile egg | *Hymenolepis nana* Egg

Giardia lamblia Cyst | *Enterobius vermicularis* Egg

Strongyloides stercoralis (male) | *Cryptosporidium parvum* Oocysts

Figure 5. Images of Human Gastrointestinal Parasites

4. Discussion

The present study focused on the contamination of sandy shore of Gaza City, the detected human gastrointestinal parasites in the sandy shore considered as evidence of contamination. It is clear that the observed discharge points reach the sandy beach are the main source for the existence of the detected intestinal parasites where moisture and suitable temperature are available. Sandy soils represents an important source of human infection by parasites, due to their geological characteristics, being formed by sand particles with diameters ranging from 0.02 to 2 mm, and with the ability to retain water between the spaces of soil particles (Rocha *et al.*, 2011).

In the same regard it is very important to mention that since wastewater treatment plants in Gaza strip are partially active, so untreated sewage is discharged to the Mediterranean Sea directly. Fathers reach sandy beaches with their children where they are digging in the sand, not knowing they will be exposed to contaminate their hands with parasites, according to the OPAS 2002 report, it is estimated that two billion people in the world are infected by some form of parasites acquired through the contact with soil, 800 million of the infected are children (40%) (Da Silva *et al*., 2012).

Very little information exists concerning the presence of viruses and parasites in the beach sand. In a three-year study in Romania by Nestor *et al.* (1984), the incidence of parasites was found to depend on season, during non-vacation seasons no parasites being present in seawater and beach sand. In a study of two sand beaches in Marseilles, France, *Toxocara canis* was found to be the most common parasite, being present on average in 150 g of sand (Signorile *et al.*, 1992). However, in a study carried out on "dog beaches" in Perth, Australia, a total of 266 samples showed no traces of *Toxocara canis* eggs or other eggs/larvae of parasitic nematodes (Dunsmore *et al.*, 1984). It was emphasized in that study that the major risk to humans was from an environment in which puppies, not older dogs, were found. The presence of other parasites transmitted by water (Marshall *et al.*, 1997) that have not been investigated in recreational sand areas may be potentially significant.

The evidence of contaminated Gaza sandy shoreline region has been supported by many studies. Beaches represent the unconsolidated sediment that lies at the junction between water (oceans, lakes and rivers) and land and are usually composed of sand, mud or pebbles. From a recreational viewpoint, sand beaches should be clear and healthy. Especially in higher latitudes, a significant percentage of time is spent on the beach itself rather than in the water. Microorganisms are a significant component of the polluted beach sand. Bacteria, fungi, parasites and viruses have all been isolated from polluted beach sand. A number of genera and species that may be encountered through contact with sand are potential pathogens. Accordingly, concern has been expressed that beach sand or similar materials may act as reservoirs or vectors of infection (Nestor *et al.*, 1984; Roses *et al.*, 1988; Mendes *et al.*, 1997) although transmission by this route has not been demonstrated in epidemiological studies.

The prevalence of parasites in wet sand (swash zone) may attribute to the nature of the sand which act as filter and cumulative tool, but the prevalence of parasites in the dry sand may be attributed to the tidal action, when seawater cover the dry sand the parasites remain in the dry sand.

As mentioned, wastewater effluent may transport faecal-associated microorganisms such as parasites, which are implicated in thousands of illnesses each year among people who consume contaminated shellfish (Burkhardt and Calci, 2000; Shieh *et al.*, 2000). There is a substantial need for better detection and identification of wastewater influences on coastal systems to inform bathers and fisheries management and protect public health (Randall, 2003; Savage, 2005).

The situation of random discharge points of sewage exists since decades, only four WWTPs present in Gaza Strip, the situation of contamination sandy beach will be worse in case of no municipal or environmental actions were taken.

The cycle of parasitic diseases still exist in Gaza Strip, and could be found within infected human, some animals, contaminated food, polluted environments and unsuitable drinking water reservoirs. Additionally, many reported

literatures were carried out in Gaza Strip confirm the endemicity of the human gastrointestinal parasites is in one of the most crowded and overpopulated area in the world. Yassin *et al*. (1999) reported that the prevalence of intestinal among school children which was 27.6%.

In a study carried out by Kanoa (2006) the impact of health education programmer's intervention on the prevalence of intestinal parasites among school children in Gaza city, B eit-lahia villages and Jabalia refugee camp "Gaza Strip" were examined for 6 months. The rule of health education in decreasing prevalence of intestinal parasitic infection was statistically significant (*P*= 0.001). A comparison study was carried out between two regions in Gaza strip, it was found that Prevalence of intestinal parasites was high in Jabalia village (more than 53%) in comparison to Rimal area 33% (Al Agha and Teodorescu, 2002). A house hold survey included 1000 individuals from all ages was conducted in Biet-lahia, Palestine It was found that (72.9%) of examined individuals were infected with different types of intestinal parasites (Al-Zain and Al Hindi, 2005).

These studies confirm the source of intestinal parasites from decades and continue to exist from the infected hosts to the sewers which find its way to the shoreline region.

5. Conclusion

It is concluded that the prevalence of parasites in wet sand is higher than in adjacent dry sand, as the wet sand behaves as a passive harbor for cumulative pollution.

6. Recommendations

It is recommended that contaminated areas should be identified and residents should be aware of such risk which is a result of the contamination of sandy Gaza sea shore, and the multiple discharge point should be grouped into one.

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In vitro Screening of *Lactobacillus* species from Homemade Yoghurt for Antagonistic Effects against Common Bacterial Pathogens

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Abstract

Four species of lactic acid bacteria (LAB) were isolated from homemade yoghurt samples and defined as *Lactobacillus acidophilus Lactobacillus bulgaricus, Lactobacillus casei*, and *Lactobacillus plantarum*. Each LAB isolate was tested for its tolerance to acidic environments at pH (7.0, 4.0, and 2.0). All lactobacilli isolated tolerated acid while *L. bulgaricus* was sensitive to pH 2.0. All four bacteria were resistant to ciprofloxacin. Isolates were further tested for their antimicrobial activity against common pathogenic bacteria such as *Escherichia coli, Klebsiella pneumoniae, Proteus mirabilis, Pseudomonas aeruginosa, Staphylococcus aureus* (MRSA), and *Bacillus subtilis* using the agar spot method. These lactic acid bacteria were found to inhibit growth of most pathogens tested. The viability of lactobacilli isolates were not affected by storage (for 6 weeks) at -20^{o}C and 4^{o}C but declined when they were stored at room temperature. Acid tolerance and bacterial antagonistic characteristics of these LAB isolates render them good candidates for consideration as probiotics.

Keywords: *Lactobacillus* species, Homemade Yoghurt, Antagonistic Effect, Pathogenic Bacteria, Commercial Probiotics.

1. Introduction

The rapid emergence of drug resistant strains of microbial pathogens, especially those with multiple drug resistances, is a major health problem because of their high occurrence worldwide (Rouveix, 2007).

Infectious diseases are the biggest problem in man. Every year gastrointestinal infections lead to significant morbidity and mortality worldwide (Culligan *et al*., 2009). The World Health Organization (WHO, 2004) estimates that more than 4 billion episodes of diarrhoeal disease occur annually, and that 2.2 million deaths were attributable to enteric infection, making it the fifth leading cause of death at all ages worldwide. Enteric bacteria comprised of *Salmonella* species, *Shigella* sp., *Proteus* sp., *Klebsiella* sp., *E. coli*, *Pseudomonas* sp., *Vibrio cholerae* and *Staphylococcus aureus*, are major etiologic agents of enteric infection (Ballal and Shivananda, 2002). The rise in antibiotic resistant bacteria has awakened the scientific community to the prophylactic and therapeutic uses of probiotics, and to reconsider them as alternatives to antibiotics (Ahmed, 2003).

Consumption of food containing live bacteria is the oldest and still most widely used way to increase the number of advantageous bacteria called "probiotics" in the intestinal tract (Salminen and Von Wright, 2011). Noteworthy, there are large number of probiotic foods that date back to ancient times. These mostly originated from fermented foods, as well as, cultured milk products (Tadesse *et al*., 2005; Kent and Hayward, 2007; Salminen and Von Wright, 2011). The quest to find food ingredients with valuable bioactive properties has created interest in lactic acid bacteria (LAB) with probiotic attributes such as antimicrobial activity against pathogenic microorganisms (Hugo *et al.*, 2006), antiviral activity (Botik, 2007), anti-yeast activity (Kantachote, 2008), and antimutagenic activity (Sung *et al.*, 2006). In addition, probiotic microorganisms have been used as a food preservative and antimicrobial agent more than other chemical agents due to the probiotic effects on human and other animal foods (Hassan *et al.*, 2013). LAB are the most prominent non-pathogenic bacteria that play a vital role in our everyday life, from fermentation, preservation, and production of wholesome foods, and vitamins, to prevention of certain diseases and cancer due to their antimicrobial action (Keith, 1991).

The majority of microorganisms used as probiotics belong to the LAB and bifidobacteria. Within the group of LAB, lactobacilli species are the most commonly utilized group of microorganisms due to their potential beneficiary properties as probiotics. The antagonistic activities of these bacteria are known to inhibit a l arge number of enteric and urinary pathogenic bacteria (Gilliland, 1990; Battcock and Azam-Ali, 1998; Hutt *et al.*, 2006).

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The use of probiotics to control certain infections has started gaining acceptance. The alarming rise of inappropriate antibiotic use, and antimicrobial resistance, along with renewed interest in ecological natural methods to prevent infections, make probiotics a very interesting field for research.

Although, there is a lot of research about antimicrobial activity and antibiotic resistance of LAB, little research was dedicated towards the isolation of LAB from homemade yoghurts. Therefore, the aim of this study was to investigate the antimicrobial activity and antibiotic susceptibility of bacteria isolated from yoghurt samples collected from several regions of Erbil city. These yoghurt bacteria were identified and tested for their resistance to acidic environments, their resistances towards some antibiotics, their antimicrobial activities against Gram-positive and Gram-negative food pathogenic and spoilage microorganisms, and their viability when stored at different temperatures.

2. Materials and Methods

2.1. Sample Collection

Due to its wide acceptance among the consumers of Erbil, homemade dairy yoghurt (known as Erbil yoghurt) was selected for isolation of lactobacilli species. The antagonistic effectiveness of these isolates was compared with commercially available probiotic capsules (Probiotane, Vitane Pharme Germany).

2.2. Isolation of Lactobacilli species from Homemade Yoghurt

A 25 g sample of homemade yoghurt was taken aseptically and homogenized in 225 ml of sterile buffered peptone water. Five 10 fold dilutions of the homogenates were then prepared and were inoculated on plates of de Man Rogosa Sharpe agar (MRS, Oxoid, England), a selective growth medium acidified with 1 N HCl to pH 5.3, and incubated anaerobically using a gas pack (Oxoid, England) for 72 hrs at 37^{o}C. Morphologically distinct and well isolated colonies were subcultured on new MRS agar plates. Finally, pure colonies were obtained.

2.3. Identification of Isolates

Identification of the lactobacilli sp. was performed according to their morphological, cultural, physiological and biochemical characteristics. Macroscopic appearance of all the colonies was examined for cultural and morphological characteristics. Size, shape, color and texture of the colonies were recorded. Isolates were characterized based on Gram's stain reaction, cell morphology, presence of capsule or endospore, motility, catalase reaction, oxidase reaction and by growth at 15^{o}C and 45^{o}C as described by Benson (1994). Tests of, nitrate reduction, sulfide, and indole production, and CO_2 from glucose and H_2S production were performed according to (Merck, 1997). Putative lactobacilli were identified to species level based on the sugar fermentation pattern of the API 20A System (bioMe´ rieux).

2.4. Tolerance to Acidic pH

Tolerance of isolated lactobacilli to acidic pH was determined by growing bacteria in acidic MRS broth. MRS broth was poured in test tubes and pH values 7.0, 4.0 and 2.0 were obtained by addition of 1M HCl or 0.5M NaOH. An amount of 5 log10 CFUs (10^5 CFUs) of each isolated lactobacilli species was inoculated in each broth tube. Test tubes were incubated at 37^{o}C for 120 minutes. Survival of lactobacilli was evaluated by a p late count method (Awan and Rahman, 2005).

2.5. Determination of Antibiotic Susceptibility of the Isolates

Six commonly used antibiotics were used to determine the antibiotic susceptibility of the isolated lactobacilli species using Kirby Bauer method. The antibiotic discs (Oxoid, England) were as follows: Amoxiclav (30µg), Tetracycline (30µg), Vancomycin (30µg), Sulfamethoxazole (100µg), Cefotaxime (10µg) and Ciprofloxacin (5µg).

2.6. Test Pathogens

Pathogenic bacterial isolates were obtained from patients associated with different infections at Rizgary and Komary Hospitals in Erbil city. These isolates were comprised of Gram-negative bacteria (*Escherichia coli, Klebsiella pneumoniae, Proteus mirabilis, and Pseudomonas aeruginosa)*, and Gram-positive bacteria (*Staphylococcus aureus (*MRSA) and, *Bacillus subtilis*). Organisms were maintained in Nutrient Agar slants and tubes at 4^{o}C.

2.7. Testing for Antibacterial Activity

Antimicrobial action of all isolated lactobacilli species and commercial probiotics against indicator pathogenic bacteria was determined using the agar spot method. The isolated LAB species and commercial probiotics were grown in MRS broth at 37^{o}C for 48-72 hrs and diluted to a density of 10^8 CFU/ml. Two microliters of each LAB species or commercial probiotics were spotted aseptically onto the MRS agar medium (which was found to support the growth of all the LAB species). The plates were then incubated anaerobically for 48-72 hrs. On the other hand, cultures of the pathogenic bacteria were prepared in nutrient broth and incubated at 37^{o}C for 16-18 hrs. They were then transferred to fresh nutrient broth and incubated at 37^{o}C for 4 hrs. The pathogenic bacterial isolates were then adjusted to 10^8 CFU/ml. One ml of each of the pathogenic bacterial isolates was then inoculated into a flask with 100 m l molten nutrient agar at 50^{o}C. Approximately 7 ml of the agar were poured as an overlay on the plate with the grown LAB, prepared as described above. The former was allowed to solidify, before being incubated aerobically at 37^{o}C for 16-18 hrs. The diameters of the clear zones (in the top agar layer) were measured to the nearest mm. The zones of inhibition were then swabbed, and the swabs were used to inoculate tubes of nutrient broth. After incubation at 37^{o}C for 24 hrs, the tubes were checked for turbidity. A clear tube indicated bactericidal activity of the LAB species, while a t urbid tube indicated bacteriostatic (Chuayana *et al.*, 2003). Each assay was done in triplicate.

2.8. Bacterial Viability During Storage

The storage viability of isolated LAB species was recorded weekly at -20^{o}C, 4^{o}C and at room temperature. The test tubes were inoculated with 10^5 CFUs of each

culture suspension. The inoculated test tubes were stored at -20°C, 4°C and room temperature for 6 weeks. The growth was monitored weekly using plate count method in MRS medium.

2.9. Statistical Analysis

All data are expressed as means of standard error (M±SE). Statistical analysis was carried out using SPSS Version 16 software. Data analysis was made using one-way analysis of variance (ANOVA). The comparisons between groups were done using Duncan post hoc analysis. *P* values less than 0.05 were considered statistically significant.

3. Results and Discussion

Increased focus has been given to food as a potential vehicle of antimicrobial substances. Such foods have become an important health care sector in most countries. Among them, are the fermented dairy products and, especially yoghurt, which are classic examples of traditional foods originating from the Middle East and Eastern Mediterranean (Tamime *et al*., 2006; Kyriacou *et al.*, 2008).

In the present study, *Lactobacillus* sp. were isolated from homemade yoghurt common to Erbil city and that is prepared from conventional milk. Based on Gram staining and various biochemical tests, four species of *Lactobacillus* were isolated, namely *L. acidophilus, L. bulgaricus, L. casei and L. plantarum.* Lactic acid bacteria (LAB) especially *Lactobacillus* species are common in milk and milk products (Mitsuoka, 1992).

Probiotic bacteria are mostly delivered in a food system and must be acid and bile tolerant to survive in the human gastrointestinal tract. The time from ingestion to passing through the stomach has been estimated to be approximately 90 minutes (Berada *et al.*, 1991).

Tolerance level to acidic environment of all species of *Lactobacillus* isolated in this study was found to be significantly variable ($p<0.05$) as expressed in table (1). *L. acidophilus, L. casei and L. plantarum* were most resistant at pH 4.0 and their viable count increased. However, at pH 2.0 their viable counts decreased. In contrast *L. bulgaricus* couldn't survive at pH 4.0 and its viable count reached zero at pH 2.0.

The pH, physical and chemical characteristics of a food carrier in which potential probiotics are relayed may have a buffering effect against gut pH, and therefore, positively influence their survival during gastric passage (Charalampopoulos *et al*., 2002; and Patel *et al.*, 2004). This may explain why *L. bulgaricus* known to exhibit poor survival when challenged in vitro to gastric acidity, showed high survival rates in the terminal ileum of fistulated minipigs fed with yoghurt (Lick *et al*., 2001). Comparable results have been reported by Liong and Shah (2005) who indicated that *L. acidophilus* and *L. casei* survived best under acidic conditions. *In vitro* survival of bacterial strains at low pH is a more accurate indication of their ability to survive passage through the stomach.

The organisms taken orally have to face stresses from the host which begin in the stomach, with pH between 1.5 and 3.0 (Corzo and Gilliland, 1999).

Table 1. Mean values (±SE) of plate count (Log10) of isolated lactobacilli at different pH values

pH	*L. acidophilus*	*L. bulgaricus*	*L. casei*	*L. plantarum*
7.0	4.33 ± 0.33^{b}	5.66 ± 0.33^{b}	5.00 ± 0.57^{b}	5.33 ± 0.33^{b}
4.0	6.66 ± 0.33^{c}	1.00 ± 0.57^{a}	6.33 ± 0.88^{b}	5.33 ± 0.88^{b}
2.0	2.73 ± 0.17^{a}	0.00 ± 0.00^{a}	2.00 ± 0.57^{a}	1.33 ± 0.33^{a}

Means having different superscripts in a column or row are significantly different ($P<0.05$).

Antibiotic resistance of the isolated lactobacilli species is summarized in table (2). The four species were sensitive towards most tested antibiotics. Notable observation is the resistance to ciprofloxacin, expressed by all isolates. Antibiotic resistance of microorganisms used as probiotic agents is an area of growing concern. It is believed that antibiotics used for food-producing animals can promote the emergence of antibiotic resistance in bacteria present in the intestinal microflora. Then, these antibiotic-resistant bacteria can transfer resistance factors to other pathogenic bacteria through the exchange of genetic material (Mathur and Singh, 2005). One of the safety considerations in probiotics is verifying that a potential probiotic strain does not contain transferable resistance genes (Temmerman *et al.,* 2003).

Table 2. Antibiotic sensitivity of the lactobacilli species isolated from yoghurt samples

Lactobacillus species	Diameter of inhibition zone in mm					
	AMC	TE	VA	SMZ	CTX	CIP
L. acidophilus	30(S)	0(R)	25(S)	35(S)	27(S)	0(R)
L. bulgaricus	32(S	30(S)	25(S)	32(S)	30(S)	0(R)
L. casei	31(S)	0(R)	25(S)	28(S)	27(S)	0(R)
L. plantarum	30(S)	27(S)	24(S)	29(S)	25(S)	0(R)

Antibiotics (Disk potency): AMC: Amoxiclav (30µg); TE: Tetracycline (30µg); VA: Vancomycin (30µg); SMZ: Sulfamethoxazole (100µg); CTX: Cefotaxime (10 µg); CIP: Ciprofloxacin (5 µg); (S): sensitive; (R): resistant.

The antagonistic effect of the isolated LAB species on some common pathogenic bacteria was evaluated using the agar-spot method. Results in table (3) showed that all LAB species exhibit antagonistic effect on both Gram-positive and Gram-negative bacteria. Statistically, there was no significant difference between the lactobacilli species regarding their antagonistic effect on pathogenic bacteria. However, when this effect was compared with that of commercial probiotics, a significant ($p<0.05$) difference was observed. All lactobacilli isolates exhibited better inhibitory effects than commercial probiotics. The *in vitro* inhibition of the pathogenic isolates by the probiotic species may have been due to the secretion of organic acids, primarily lactic acid, which decreases the pH of the medium to a level unsuitable for growth (Marianelli *et al.*, 2010).

Table 3. *In vitro* antagonistic effect of the *Lactobacillus* sp. on the pathogenic bacteria and the mean (±SE) diameters of zones of inhibition (in mm) in the agar spot assay

Test pathogens	*L. acidophilus*	*L. bulgaricus*	*L. casei*	*L. plantarum*	Commercial Probiotic
Escherichia coli	24.83±0.60[a]	24.00±2.08[a]	27.33±4.05[a]	25.00±0.57[a]	20.00±2.88[b]
	Bactericidal	Bactericidal	Bactericidal	Bactericidal	Bactericidal
Klebsiella pneumoniae	27.33±3.84[a]	27.33±1.76[a]	27.00±1.52[a]	28.00±3.05[a]	20.66±.66[b]
	Bactericidal	Bactericidal	Bactericidal	Bactericidal	Bactericidal
Proteus mirabilis	25.00±2.88[a]	28.33±2.40[a]	28.33±4.40[a]	27.00±1.73[a]	20.66±2.96[b]
	Bactericidal	Bactericidal	Bactericidal	Bactericidal	Bactericidal
Pseudomonas aeruginosa	30.00±2.88[a]	27.00±1.52[a]	27.66±3.71[a]	26.33±0.88[a]	22.66±1.45[b]
	Bactericidal	Bactericidal	Bacteriostatic	Bacteriostatic	Bactericidal
Staphylococcus aureus (MRSA)	25.00±2.88[a]	26.66±2.02[a]	28.00±2.30[a]	25.00±2.88[a]	21.33±0.66[b]
	Bactericidal	Bactericidal	Bacteriostatic	Bactericidal	Bactericidal
Bacillus subtilis	23.33±1.66[a]	27.00±2.51[a]	27.33±1.45[a]	29.00±3.21[a]	23.66±0.66[b]
	Bactericidal	Bactericidal	Bactericidal	Bactericidal	Bactericidal

Means having different superscripts in a column or row are significantly different (P<0.05).

Different reports showed that most lactobacilli strains produce substances that inhibit pathogenic, non-pathogenic and spoilage organisms in fermenting foods and beverages. In general, the antimicrobial activity of lactobacilli may be due to lactic acid, acetic acid, formic acid, phenyllactic acid, caproic acid, organic acids, ethanol organic acids, hydrogen peroxide, bacteriocins or other inhibitory metabolites. Lactic acid and acetic acid are particularly important compounds, inhibiting a broad range of microorganisms (Helander *et al*., 1997). Yoghurt bacteria are especially effective for the prevention and treatment of some diseases mediated by pathogenic microorganisms through several mechanisms, such as the production of the above mentioned substances (which are active at low pH). In addition, they are able to eliminate the colonization with pathogenic bacteria and treat gastrointestinal tract infections (Petti *et al*., 2008).

It is especially worth noting that most of the antimicrobial activities exhibited by the probiotics were bactericidal in nature, with the exception of *L. casei* which was bacteriostatic against both *Staphylococcus aureus* and *Pseudomonas aeruginosa*. This is consistent with an earlier investigation which showed that the bacterium isolated from yoghurt and identified as *L. casei* was bacteriostatic against methicillin susceptible *Staphylococcus aureus* and *Pseudomonas aeruginosa* (Chuayana *et al*., 2003).

The viability of the *Lactobacillus* species was investigated during storage at three different temperatures, the results demonstrated that storage at -20°C and 4°C had no effect (p<0.05) on the viable count of all isolates even after 6 weeks of storage as shown in tables 4 and 5. A significant (p<0.05) decline in viable count was observed for all isolates at room temperature after 6 weeks of storage as indicated table 6. Low temperature storage of yoghurt enables use of these LAB species as probiotics. These results agree with that of Ashrad *et al*. (2009).

Table 4. Mean values (M±SE) of viable count (Log10) of isolated lactobacilli during storage at -20°C.

Weeks of storage	*L. acidophilus*	*L. bulgaricus*	*L. casei*	*L. plantarum*
1st	4.63±0.44	4.33±0.33	4.33±0.33	4.50±0.28
2nd	4.46±0.29	4.33±0.33	4.50±0.28	4.50±0.50
3rd	4.66±0.16	4.33±0.33	4.16±0.44	4.33±.88
4th	4.33±0.33	4.00±1.15	4.33±0.33	4.33±0.63
5th	4.33±0.33	4.00±0.57	4.33±0.88	4.50±0.28
6th	4.00±0.57	4.33±0.33	4.66±0.33	4.66±0.33

Table 5. Mean values (M±SE) of viable count (Log10) of isolated lactobacilli during storage at 4°C

Weeks of storage	*L. acidophilus*	*L. bulgaricus*	*L.casei*	*L. Plantarum*
1st	4.63±0.44	4.33±0.33	4.33±0.33	4.50±0.28
2nd	4.46±0.29	4.33±0.33	4.50±0.28	4.50±0.50
3rd	4.66±0.16	4.33±0.33	4.16±0.44	4.33±.88
4th	4.33±0.33	4.00±1.15	4.33±0.33	4.33±0.63
5th	4.33±0.33	4.00±0.57	4.33±0.88	4.50±0.28
6th	4.00±0.57	4.33±0.33	4.66±0.33	4.66±0.33

Table 6. Mean values (M± SE) of viable count (Log10) of isolated lactobacilli during storage at room temperature.

Weeks of storage	*L. acidophilus*	*L. bulgaricus*	*L. casei*	*L. plantarum*
1st	4.80±0.35[b]	4.63±0.08[c]	4.43±0.23[b]	4.66±0.06[d]
2nd	4.56±0.12[b]	4.70±0.05[c]	4.60±0.05[b]	4.66±0.06[d]
3rd	4.46±0.31[b]	4.46±0.24[c]	4.50±0.32[b]	4.03±0.31[c]
4th	4.43±0.29[b]	3.66±0.12[b]	3.60±0.15[a]	3.70±0.15b[c]
5th	3.50±0.28[a]	3.56±0.38[b]	3.50±0.15[a]	3.40±0.11[b]
6th	3.30±0.35[a]	2.73±0.14[a]	2.96±0.41[a]	2.80±0.11[a]

Means having different superscripts in a column or row are significantly different (P<0.05).

4. Conclusion

Development of resistance to antibiotics by bacteria is inevitable, not only because of their high rates of mutation and transferability of drug resistance genes, but also because antibiotics pose a selective pressure against these bacteria, prompting drug-resistant strains to out compete the susceptible ones (Pray, 2008). The rise of multidrug resistant strains has therefore, led researchers to look for alternative therapies such as probiotics that would decrease our reliance on a ntibiotic use. It is concluded from the present study that species of *Lactobacillus* isolated from homemade yoghurt have the ability to survive at low pH and low temperatures and had strong antagonistic effects against several pathogenic bacteria. These results also suggest that the consumption of products containing probiotics can protect an individual from developing infections caused by pathogenic bacteria. Therefore, probiotics may be helpful in addressing the worldwide issue of antibiotic resistance.

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Bioefficacy of Azadirachtin in Controlling *Culex pipiens pipiens* (Diptera: Culicidae)

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Abstract

Azadirachtin was tested for its effects against First and second instars larvae of mosquito *Culex pipiens pipiens* (Diptera: Culicidae), laboratory reared larvae were exposed to 0.125 ; 0.250; 0.500 and 0.750 mg/ L of azadirachtin in laboratory of biology animal application, biology Department , university of Badji Mokhtar, Annaba, Algeria. Larvicidal assays were conducted according to standard World Health Organization (WHO) .The results have been exploited according to classic statistical methods. A linear correlation was revealed between concentration and larval mortality. At first stage, larval mortality increased from 45.83 % at 0.125 mg /L to 94.44 % at concentration 0.750mg /L of Azadirachtin in direct effect. The lethal concentration LC_{16}, LC_{50} and LC_{90} in direct effect was measured as 0.056; 0.166 and 0.663 mg/L respectively. Cumulate mortality increased from 54.28% to 95.71% at 0.125mg/L and 0.750mg/L respectively. The LC_{16}, LC_{50} and LC_{90} values for Azadirachtin were 0.040 ; 0,127 and 0.555mg/L respectively. At second stage, larval mortality increased from 39.66 % at 0.125 mg /L to 87.66 % at concentration 0.750mg /L of azadirachtin in direct effect, the LC_{16}, LC_{50} and LC_{90} values was 0.063; 0.190 and 0.891 mg/L respectively. In indirect effect the mortality increased from 49.27% to 91.54% at 0.125mg/L and 0.750mg/L respectively, the LC_{16}, LC_{50} and LC_{90} values was 0.041; 0.141 and 0.724 mg/L respectively. After a comparison between the two stages showed that the first stage is the most sensitive than the second stage .The results show that the azadirachtin is promising as a larvicidal agent against *Culex pipiens pipiens* naturally occurring biopesticide could be an alternative for chemical pesticides.

Keywords: Mosquito, *Culex pipiens pipiens,* Azadirachtin, Insecticide.

1. Introduction

Various neem products have been investigated extensively for their phytochemistry and exploitation in pest control programmes. A number of bioactive components have been isolated from various parts of the neem tree *(Azadirachta indica:* Meliacae*).* The Meliaceae plant family is known to contain a variety of compounds, which show insecticidal, antifeedant, growth regulating and development modifying properties (Greger *et al*., 2001; D'Ambrosio and Guerriero, 2002; Nakatani *et al*., 2004). The effects of the compounds extracted from *M. azedarach* on insects have been reviewed by Ascher *et al*., (1995) and reported by Saxena *et al*., (1984), Schmidt *et al*., (1998), Juan *et al*., (2000), Carpinella *et al*., (2003) , Senthil Nathan and Saehoon, (2005). Control of mosquito is essential as many species of mosquitoes are vectors of malaria, filariasis, and many arboviral diseases and they constitute an intolerable biting nuisance (Collins and Paskewitz, 1995). The development of insect's growth regulators (IGR) has received considerable attention for selective control of insect of medical and veterinary importance and has produced mortality due to their high neurotoxic effects (Wandscheer *et al*., 2004; Senthil Nathan *et al*., 2005a). Although, biological control has an important role to play in modern vector control programs, it lacks in the provision of a complete solution by itself. Irrespective of the less harmful and ecofriendly methods suggested and used in the control programmes, there is still need to depend upon the chemical control methods in situations of epidemic out break and sudden increase of adult mosquitoes. Recent studies stimulated the investigation of insecticidal properties of plant derived extracts and concluded that they are environmentally safe, degradable, and target specific (Senthil Nathan and Kalaivani, 2005). Muthukrishnan and Puspalatha (2001) evaluated the larvicidal activity of extracts from *Calophyllum inophyllum* (Clusiaceae), *Rhinacanthus nasutus* (Acanthaceae), against *Anopheles stephensi* (Senthil Nathan *et al*., 2006) were studied for their larvicidal action on fourth instar larva of *Culex quinquefasciatus* (Kalyanasundaram and Dos, 1985). Murugan and Jeyabalan (1999) reported that *Leucas aspera*, *O. santum*, *Azadirachta. indica*, *Allium sativum* and *Curcuma longa* had strong larvicidal, anti-emergence,

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adult repellency and anti-reproductive activity against *A. stephensi*. In addition, *Pelargonium citrosa* (Jeyabalan *et al*., 2003), *Dalbergia sissoo* (Ansari *et al*., 2000a) were shown to contain larvicidal and growth inhibitory activity against *A. stephensi.* With these backgrounds this present study was aimed to assess the larvicidal activity of azadirachtin on first and second instar larvae of *Culex pipiens pipiens* under laboratory condition.

2. Materials and Methods

2.1. Mosquitoes

Culex pipiens pipiens eggs were collected from cellarage tribes (region sidi amar - Annaba) and readed in the laboratory of biology animal application, university of Annaba- Algeria. Larvae were placed in Pyrex storage jars (80 by 100mm) containing 200 ml of tap water. They were maintained at 25-27°C, 75-85% relative humidity under 14:10 light and dark photo period cycle. The larvae were fed with fresh food consisting of a mixture of Biscuit Petit Regal-dried yeast (75:25 by weight). Pupae were transferred from the trays to a cup containing tap water and placed in screened cages (20x20x20cm) where the adult emerged. After emergence, female mosquitoes obtained blood meal from caged pigeons while male mosquitoes were fed on a 10% sucrose solution. Then egg-masses were kept to continue next generation.

2.2. Laboratory Bioassay procedure

The selected insecticides were evaluated against the against the 1st and 2nd instars larvae of mosquito *Culex pipiens pipiens (Diptera: Culicidae)* using the standard bioassay technique (WHO). The Bioassays were performed with using concentration from 0.125; 0,250; 0,500 and 0,750mg/L of the azadirachtin. For mortality studies, 25 larvae of 1st and 2nd instars were introduced in Pyrex storage jars (80 by 100mm) containing various concentrations of the azadirachtin. A control was maintained. The treatments were replicated five times and each replicate set contained one control. The percentage mortality was calculated by using the formula (1) and corrections for mortality when necessary were done using Abbot's (1925) formula (2).

$$\text{Percentage of mortality} = \frac{\text{Number of dead larva}}{\text{Number of larvae introduced}} \times 10 \quad (1)$$

$$\text{Corrected percentage of mortality} = 1 - \frac{n \text{ in T after treatment}}{n \text{ in C after treatment}} \times 100 \quad (2)$$

Where n = number of larvae, T = treated, C = control.

2.3. Statistical Analysis

For larval bioassay under laboratory conditions, the differences between the LC_{16}, LC_{50} and LC_{90} values are considered significant if their fiducial limits (95%) did not overlap as mentioned by Litchfield and Wilcoxin (1949).

In addition, statistical analysis was carried out for all the estimated measurements of treatments and compared with the control values by test ANOVA and Student's *t*-test using the computer program (MINITAB, version 13).

3. Results

3.1. Insecticidal activity

Results of treating early the first and second instar larvae of *C. pipiens pipiens* with different concentrations follow: 0.125; 0.25; 0.50 and 0.75 mg/L of azadirachtin exhibited insecticidal activity with a dose – response relationship. Moreover, this compound presented toxicity by direct action on the treated larval instars but also by differed action (cumulate mortality) on t he other following stages of development

3.1.1. Effect Direct

For first stage, the highest concentration tested 0,750mg/L in direct effect, caused 94,44% mortality and under concentration caused 45,83% mortality presented in Figure 1. With probit, the regression equation as Y = 2,13X+0,90, the LC_{50} was calculated as 0,166 mg/L (95% Cl=0,139 – 0,197 mg/L), LC_{16} as 0,056mg/L and LC_{90} was 0,663 mg/L presented in Table 2. After treatment the second stage, in direct effect, the highest concentration, caused 87,66% mortality and less concentration caused 39.66% mortality of larvae (Figure 3) , the LC_{50} was 0,190 mg/L (95% Cl=0,157-0,230 mg/L) , LC_{16} as 0,063 mg/L and the LC_{90} was 0,891 mg/L presented in Table2. After a comparison between the two stages showed that the first stage is the most sensitive than the second stage, because the percentage of mortality at the first stage is high of mortality at the second stage, and the LC_{50} and LC_{90} of first stage is less that the LC_{50} and LC_{90} of second stage presented in Figure 5.

3.1.2. Effect Indirect (Cumulated Effect)

Dose response relationship was determined for azadirachtin applied for first and second instar larvae, the mortality was scored up t o adult formation. After treatment the first stage, the highest concentration tested 0,750 mg/L in indirect effect, caused 95,71% mortality and under concentration caused 54,28% mortality, presented in Figure 2. The LC_{50} was calculated as 0,127 mg/L (95% Cl=0,106-0,152 mg/L), LC_{16} as 0.040mg/L and the LC_{90} was 0,555 mg/L, presented in table1. After treatment the second stage, in indirect effect, the highest concentration, caused 91,54% mortality and less concentration caused 49,27% mortality of larvae (Figure 4) , the LC_{50} was 0,141 mg/L (95% Cl=0,114-0,173mg/L) , LC_{16} as 0,041mg/L and the LC_{90} was 0,724 mg/L presented in Table2. After a comparison between the two stages showed that the first stage is the most sensitive than the second stage, because observes an increase of mortality always of the first larval stage compared to the second stage and lethal concentrations at the first stage is less that the second stage, presented in Figure 6.

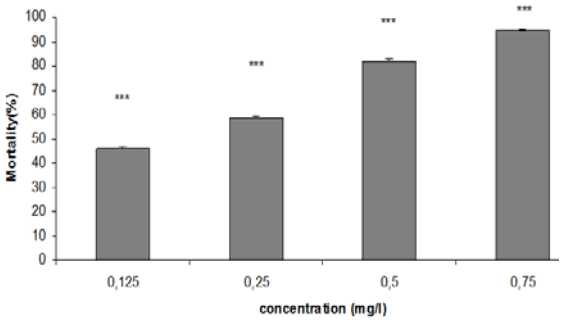

Figure 1. Larvicidal activity of azadirachtin against the first instars larvae of *Culex pipiens pipiens (effect direct)* (data following by *** are significantly different to concentrations, $p \leq 0.001$)

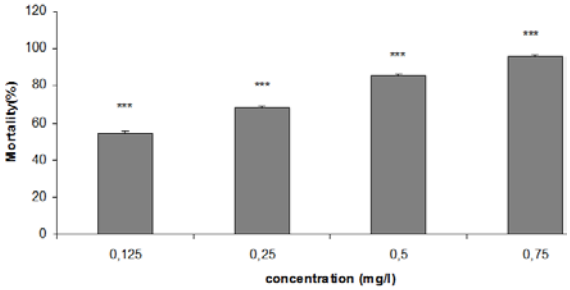

Figure 2. Larvicidal activity of azadirachtin against the first instars larvae of *Culex pipiens pipiens (effect indirect)* (data following by *** are significantly different to concentrations, $p \leq 0.001$).

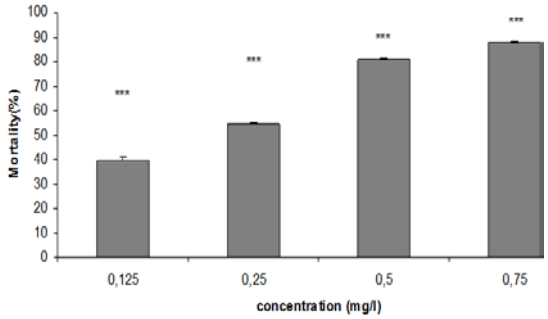

Figure 3. Larvicidal activity of azadirachtin against the second instars larvae of *Culex pipiens pipiens (effect direct)* (data following by *** are significantly different to concentrations, $p \leq 0.001$)

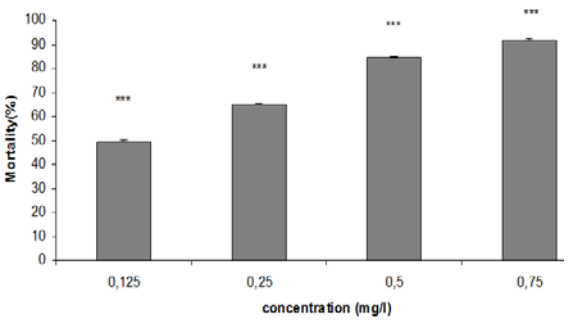

Figure 4. Larvicidal activity of azadirachtin against the second instars larvae of *Culex pipiens pipiens (effect indirect)* (data following by *** are significantly different to concentrations, $p \leq 0.001$)

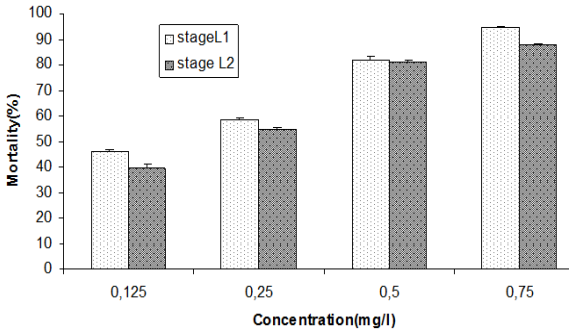

Figure 5. Effect of the azadirachtin on the two stages: comparison of mortality between the first and second instars larvae of *Culex pipiens pipiens (effect direct)*

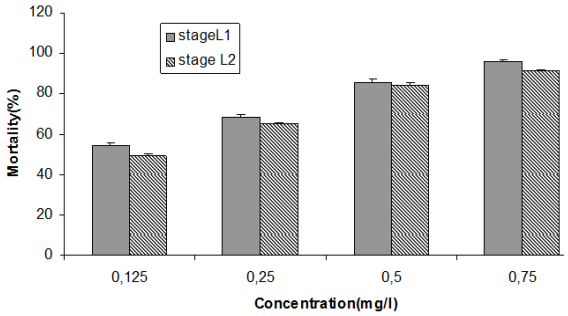

Figure 6. Effect of the azadirachtin on the two stages: comparison of mortality between the first and second instars larvae of *Culex pipiens pipiens (effect indirect).*

Table 1. Larvicidal activity of azadirachtin at various concentrations applied on first instar larvae of *Culex pipiens pipiens* at 24 hrs exposure period.

Effects	LC_{50} (mg/l)	95% Confidence limits(mg/l)		LC_{16} (mg/l)	LC_{90} (mg/l)	Regression equation	χ^2
		Lower	Upper				
Direct	0,166	0,139	0,197	0,056	0,663	Y=2,13X+0,27	2.54
Indirect	0,127	0,106	0,152	0,040	0,555	Y=2,00X+0,79	0.81

Table 2. Larvicidal activity of azadirachtin at various concentrations applied on second instar larvae of *Culex pipiens pipiens* at 24 hrs exposure period.

Effects	LC_{50} (mg/l)	95% Confidence limits(mg/l)		LC_{16} (mg/l)	LC_{90} (mg/l)	Regression equation	χ^2
		Lower	Upper				
Direct	0,190	0,157	0,230	0,063	0,891	Y=1,89X+0,69	4.11
Indirect	0,141	0,114	0,173	0.041	0,724	Y=1,80X+1,13	2.22

4. Discussion

In the present study azadirachtin have displayed varied toxicity on first and second instar larvae of *Culex pipiens pipiens.* The results showed that an increase in mortality with the increase in concentration and the early instar larvae are much susceptible than the later ones.

Neem products are capable of producing multiple effects on a number of insect species, such as antifeeding effects, growth regulation, fecundity suppression and sterilization (Mulla and Su, 1999; Vatandoost and Vaziri, 2004; Kondo *et al.*, 2004). Azadirachtin proved to be highly efficient to larva of *C. pipiens pipiens.* At first stage, larval mortality increased from 45,83 % at 0,125 mg /L to 94,44 % at concentration 0,750mg /L of Azadirachtin in direct effect. The lethal concentration LC_{16}, LC_{50} and LC_{90} in direct effect was measured as 0,056; 0,166 and 0,663 mg/L respectively. Cumulate mortality increased from 54,28% to 95,71% at 0,125mg/L and 0,750mg/L respectively. The LC_{16}, LC_{50} and LC_{90} values for Azadirachtin were 0,040; 0,127 and 0,555mg/L respectively.At second stage, larval mortality increased from 39.66 % at 0,125 mg /L to 87,66 % at concentration 0.750mg /L of azadirachtin in direct effect, the LC_{16}, LC_{50} and LC_{90} values was 0,063; 0,190 and 0,891 mg/L respectively. In indirect effect the mortality increased from 49,27% to 91,54% at 0,125mg/L and 0.750mg/L respectively, the LC_{16}, LC_{50} and LC_{90} values was 0,041; 0,141 and 0,724 mg/L respectively. However, the results which reflect the high toxicity of azadirachtin to the developmental stages (larva, pupa and adult). The results confirmed other studies concerning this compound (Alouani *et al*., 2009). In insecticidal experiment conducted on mosquitoes with compounds extracted from *Az. Indica* using a co mmercial preparation Neemarin showed mortality for fourth instar larvae of *An. stephensi*, with LC_{50} values of 60 and 43 ppm, respectively (Ruskin, 1992) .This compares with the LC_{50} in our study of 0.184 and 0.125 mg /Liter respectively for first instar larvae of *C. pipiens pipiens.* Our results were comparable with findings from other researchers as shown. The variation in LC_{50} is due to mosquito species, formulation, climate and method of application. Neem extracts act like insect growth regulators, so the mortality at different stages were considered. Mortality of the pupae stage was significantly higher than the larvae and adult stages. In addition, the mortality of *Cx. quinquefasciatus* was significantly lower than *An. Stephensi* (Vatandoost and Vaziri, 2004). In another study, Ndung'u *et al.* (2004) reported that (LC_{50} =57,1 mg/Liter) of Azadirachtin when tested against larvae of *Anopheles gambiae* (Essam *et al*., 2006). Azadirachtin the extract of neem tree, tested in the present study is reported to be eco-friendly and not toxic to vertebrates (Al- Sharook *et al*., 1991). It is clearly proved that crude or partially purified plant extract are less expensive and highly efficacious for the control of mosquitoes rather than the purified compounds or extract (Jang *et al*., 2002; Cavalcanti *et al*., 2004). The larvae of a number of mosquito species (*Aedes spp., Anopheles spp*.) are sensitive to neem products and show antifeedant and growth regulating effects (Zebitz, 1987; Murugan *et al*., 1996). The effect of these crude plant extract on the biology, reproduction and adult emergence of the mosquitoes are remarkably greater than those reported for other plant extracts in the literature. For example 50% inhibition of the emergence of the adult mosquitoes was observed by the use of *C. inophyllum*, *S. suratense*, *S. indica* and *Rhinocanthus nasutus* leaf extracts (Muthukrishnan and Puspalatha, 2001). Similarly 88% of the adult mortality was observed by the use of *P. citrosa* leaf extracts at 2% concentration (Jeyabalan *et al*., 2003). The Meliaceae plant family is used a g rowth regulator against many insect pests (Saxena *et al*., 1984; Jacobson, 1987; Schmutterer, 1990; Gajmer *et al*., 2002; Banchio *et al*., 2003; Wandscheer *et al*., 2004).

The growth regulatory effect is the most important physiological effect of *M. azedarach* on insects. It is because of this property that family Meliaceae has emerged as a potent source of insecticides. The results of this study indicate the plant-based compounds such as azadirachtin (compounds present in the Meliaceae plant family seed) may be effective alternative to conventional synthetic insecticides for the control of *Culex pipiens pipiens* Undoubtedly, plant derived toxicants are a valuable source of potential insecticides. These and other naturally occurring insecticides may play a more prominent role in mosquito control programs in the future (Mordue and Blackwell, 1993). The results of this study will contribute to a great reduction in the application of synthetic insecticides, which in turn increase the opportunity for natural control of various medicinally important pests by botanical pesticides. Since these are often active against a limited number of species including specific target insects, less expensive, easily biodegradable to non-toxic products, and potentially suitable for use in mosquito control programme (Alkofahi *et al*., 1989), they could lead to development of new classes of possible safer insect control agents.

Plant allelochemicals may be quite useful in increasing the efficacy of biological control agents because plants produce a large variety of compounds that increase their resistance to insect attack (Berenbaum, 1988; Murugan *et al*., 1996; Senthil Nathan *et al*., 2005a). Recently, bio-pesticides with plant origins are given for use against several insect species especially disease- transmitted vectors, based on the fact that compounds of plant origin are safer in usage, without phytotoxic properties; also leave no s cum in the environment (Schmutterer, 1990; Senthil Nathan *et al*., 2004, 2005a, b).

The intensive use of pesticides produces side effects on many beneficial insects and also poses both acute and chronic effects to the *milieu* (Abudulai *et al*., 2001). The most interesting observation in the present study was the deformations observed in the Azadirachtin treatment larvae, pupa and adult of *C. pipiens pipiens* are in accord with the characteristic manifestation of exposure to other insect growth regulators and insect growth inhibitors such Flucycloxuron (Andalin), and Triflumuron (Alsystin) realized at the same conditions for treatment and laboratory conditions (Rehimi and Soltani, 1999). The present study clearly proved the efficacy of Azadirachtin on larvae, of *Culex pipiens pipiens* further studies such as mode of action, synergism with the biocides under field condition are needed.

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Micro and Macronutrient Properties of *Pleurotus ostreatus* (Jacq: Fries) Cultivated on Different Wood Substrates

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Abstract

The micro and macronutrient content of *Pleurotus ostreatus* cultivated on different woody substrates viz; *Pycnanthus ongoleubis, Ceiba pentandra* and *Cananium* sp. was investigated. Mineral analysis revealed that phosphorus had the highest value in all the minerals sampled and it value ranges between 51.97 mg/100 g to 56.77 mg/100 g while Magnesium recorded the least value of 1.69 mg/100g to 3.57 mg/100 g. *Pleurotus ostreatus* cultivated on woody substrate, *Pycnanthus ongoleubis* had higher and significantly different ($P \leq 0.05$) mineral content when compared with the other woody substrates, *Ceiba pentandra* and *Cananium* sp. The result of the proximate composition showed that *Pycnanthus ongoleubis* is the most suitable substrate for the cultivation of the *Pleurotus* mushroom based on the protein content. Amino acid analysis revealed that glutamic acid (9.01g/100 g to 10.3 g/100 g) was the most abundant amino acid in *P. ostreatus*. *P. ostreatus* cultivated on *Pycnanthus ongoleubis* had higher and significantly different ($P \leq 0.05$) values in all the amino acids when compared with *P. ostreatus* cultivated on the other woody substrates except phenlyalanine. Conclusively, *Pycnanthus ongoleubis* showed good potential as substrate for cultivation based on higher and significantly different ($P \leq 0.05$) mineral and proximate contents found in *P. ostreatus* cultivated on it.

Keywords: Woody, Substrate, *P. ostreatus*, Micro and Macronutrients.

1. Introduction

For centuries, mushrooms have been appreciated as sources of food nutrients and pharmacologically important compounds useful in medicine (Sagakami *et al*., 1991). In Eastern Countries like China and Japan, the knowledge of the use of edible and medicinal mushrooms had been passed on from one generation to the other in documented form (Oyetayo, 2011). For example, over 2,500 years ago, many medicinal mushrooms had been recorded and depicted in the earliest Chinese material medica book, *Shennong Bencao Jing,* and other succeeding Chinese medical book (Zhu, 2009). However, in most parts of Africa, consumption of mushrooms by many people is based on their organolleptic properties such as aroma, taste, flavour and texture and not on the nutritional and medicinal properties (Osemwegie *et al*., 2006).

Pleurotus species are edible mushrooms commonly known as oyster mushrooms. *Pleurotus* species contain high amounts of -amino butyric acid (GABA) and ornithine. GABA is a nonessential amino acid that functions as a neurotransmitter whereas ornithine is a precursor in the synthesis of arginine (Manzi *et al*., 1999). They grow widely in the tropical and subtropical rainforests (Chirinang and Intarapichet, 2009). Oyster mushroom cultivation has increased tremendously throughout the world during the last few decades (Chang, 1999). Oyster mushroom accounted for 14.2 % of the total world production of edible mushroom in 1997 (Chang, 1999). These mushrooms can be used industrially for mycoremediation purposes. Hence, its cultivation can play an important role in managing organic wastes whose disposal has become a problem (Das and Mukherjee, 2007). The cultivation of oyster mushrooms is on any type of ligno cellulose material like straw, sawdust, rice hull and others. Presently sawdust is commonly used and is the preferred medium at commercial scale. Of the sawdust types, softwood sawdust like mango and cashew are known to be more suitable than hardwood sawdust. Hami (1990) studied different species of mushroom cultivated on sawdust of different woods and found that *P. ostreatus* gave the maximum yield.

The different substrates used in cultivating mushrooms do have effect on the functional, organoleptic and chemical properties of mushrooms. In a study, Michael *et al*. (2011) reported that protein, ash, iron and phosphorus contents were high for mushrooms grown on bean straw compared to wheat straw. In the tropics, *Pleurotus* species

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usually grow on some woody substrates in the wild. Some of these tropical woods include *Canarium* sp., *Pycnanthus ongoleubis* and *Ceiba pentandra*. The present study therefore seeks to assess the effect of different woody substrates on the mineral and proximate contents of *Pleurotus ostreatus*.

2. Materials and Methods

2.1. Preparation and Cultivation of Pleurotus ostreatus on Woody Substrate

A modified method of Chang and Miles (2004) for cultivating mushrooms was adopted. Briefly, substrates used in cultivating *Pleurotus ostreatus* were prepared from sawdust of tropical plants, *Canarium* sp., *Pycnanthus ongoleubis* and *Ceiba pentandra* mixed with water. The substrates were filled into polythene bags (800g/bag). The bags were then sealed, autoclaved, cooled and inoculated with the spawn of *Pleurotus ostreatus* collected from Federal Institute of Industrial Research Oshodi, Lagos, Nigeria. Sawdust substrate in bags were inoculated with approximately 2 g of spawn using surface spawning technique under laminar flow and incubated in a dark chamber.

The growth of mycelium in each bag was observed. When the mycelium fully covered the substrate, bags were kept open in the growing house for fruit body formation. The harvested mushroom fruit bodies were air-dried after which they were ground to powder using grinding machine.

2.2. Determination of Mineral Content of Cultivated Mushroom

The mineral composition of cultivated *P. ostreatus* was determined by wet-ashing method. The solution of ash obtained from the cultivated *P. ostreatus* samples above were dissolved in a drop of Trioxonitrate (V) acid made up to 50ml with deionised water and analysed for Calcium (Ca) and Magnesium (Mg) using atomic absorption spectrophotometer (Buck 201 VGP). Sodium (Na) and Potassium (K) were analysed using flame photometer while Phosphorus (P) was determined in the samples using UV-Visible spectrophotometer after making ammonium vanadate molybdate complex at 436nm using established procedures of Perkin-Elmer (1982).

2.3. Analysis of Proximate Composition of Cultivated Pleurotus ostreatus

Analyses were carried out on triplicate samples. The protein, ash, fat, moisture and crude fibres were determined by the method of AOAC (2000). Carbohydrate content were determined by difference i.e. (%CHO = 100-(% Ash + % protein + % fat + % moisture).

2.4. Determination of Amino Acid Content of Cultivated Pleurotus ostreatus

Each fresh mushroom sample (0.2–1 g) was hydrolysed under nitrogen gas with 15 ml of 6 N HCl in an autoclave at 110 °C for 24 h and neutralized to pH 7.00 by 4 N NaOH.. The amino acids in the hydrolysates were analysed using Technicon sequential multisample (TSM) amino acid analyzer according to the method of Spackman *et al*. (1958).

2.5. Statistical Analysis

All experiments were carried out in triplicates. Data obtained were analyzed by one way analysis of variance and means were compared by Least Significant Difference (LSD) tests (SPSS 11.5 version). Differences were considered significant at $p<0.05$.

3. Results and Discussion

Pleurotus species also known as oyster mushrooms can be cultivated on different cellulosic waste. The different substrates used in cultivating these mushrooms obviously have significant effect on the nutritional and functional properties of oyster mushroom. The present study reports the mineral and proximate contents of *P. ostreatus* cultivated on different tropical woody substrates.

The mineral content of *P. ostreatus* cultivated on different woody substrate is shown on T able 1. Phosphorus was the most abundant mineral element in the cultivated mushroom samples. The value ranges between 51.97 mg/100g to 56.77mg/100g while Magnesium recorded the least value of 1.69mg/100g to 3.57mg/100g. *Pleurotus ostreatus* cultivated on *Pycnanthus ongoleubis* recorded highest values while *Pleurotus ostreatus* cultivated on *Ceiba pentadra* recorded the lowest values in all minerals analyzed. In an earlier study, phosphorus and potassium were reported as the most abundant mineral elements in *P. sajor-caju* fruitbodies cultivated on corncobs (Oyetayo, 2005). Manzi *et el*., 1999) also reported potassium as the most concentrated mineral element in various species of edible mushrooms. The variations in the levels of elemental composition of these mushrooms may be due to the accumulation and adsorption of these elements from the substrates.

The proximate composition of *Pleurotus ostreatus* cultivated on woody substrates is shown on Table 1. The moisture content of dried *Pleurotus ostreatus* ranges from 9.00% to 10.72%. Chang and Miles (2004) had earlier reported the moisture content of dried mushrooms to be in the range 9 - 13%. The protein composition of the samples ranges between 20.03 to 20.11%. The protein content in this study is in the range of protein (20.28%) reported for *Pleurotus ostreatus* cultivated on cotton waste (Bonatti *et al.,* 2004). Comparatively, *Pleurotus* cultivated on *Pycnanthus ongoleubis* had the highest protein content (20.11%). The crude fibre obtained in this study ranges between 17.35-17.51%. This is lower than crude fibre content (18.50%) reported for *Pleurotus* cultivated on cotton waste (Bonatti *et al.,* 2004). *Pleurotus* cultivated on *Pycnanthus ongoleubis* had the highest crude fibre. Fat content (2.31% to 3.09%) obtained in the present study was found to be low which is in correlation with the characteristics of oyster mushroom reported by Manzi *et al*. (1999). *Pleurotus* species are known to have low fat content (Akindahunsi and Oyetayo, 2006). *Pleurotus* cultivated on *Ceiba pentandra* had a lowest fat content (2.31%).

The carbohydrate content was higher in the *Pleurotus ostreatus* cultivated on *Pycnanthus ongoleubis* (45.75%)

than the *Pleurotus* cultivated on *Ceiba pentandra* and *Cananium* sp (41.87% and 44.56% respectively) but lower than the *Pleurotus* cultivated on cotton waste (48.35%). This is similar to the report of Ragunathan and Swaminathan (2000) that carbohydrate content of *Pleurotus ostreatus* ranged between 40.60-53.30%. There was a great variation in the ash content result of the samples. *Pleurotus ostreatus* cultivated on *Ceiba pentandra* wood substrate recorded the highest value (8.19%) and *Pleurotus ostreatus* cultivated on *Pycnanthus ongoleubis* recorded the least value (4.75%). The result of the proximate composition determination showed that *Pycnanthus ongoleubis* is the most suitable substrate for the cultivation of the *Pleurotus* mushroom based on the protein content.

Table 1. Mineral and Proximate composition of *Pleurotus ostreatus* cultivated on different woody substrates

Composition	A	B	C
Potassium(mg/100g)	11.34^{c}± 0.02	9.42^{a}± 0.15	10.33^{b}± 0.025
Sodium (mg/100g)	4.39^{c}± 0.012	4.03^{a} ± 0.02	4.11^{b} ± 0.01
Calcium (mg/100g)	8.87^{c} ± 0.006	5.37^{a} ± 0.01	6.85^{b} ± 0.017
Magnesium(mg/100g)	3.57^{c}± 0.01	1.69^{a}± 0.015	2.22^{b}± 0.015
Phosphorus (mg/100g)	56.77^{c} ±0.015	51.97^{a} ±0.01	53.24^{b}± 0.04
Ash (%)	4.75^{a} ±0.05	8.19^{c} ±0.01	6.76^{b} ± 0.03
Protein (%)	20.11^{c} ±0.05	20.03^{a}± 0.017^{a}	20.06^{b} ±0.02
Carbohydrate (%)	45.74^{b} ±0.06	41.8± 0.05	45.74^{b} ±0.06
Moisture Content (%)	9.25^{b} ± 0.03	10.72^{c} ±0.03	2.22^{b}± 0.02
Fat (%)	3.09^{c} ±0.02	2.31^{a} ±0.02	2.76^{b} ± 0.05
Dietary Fibre (%)	17.51^{c} ±0.02	17.35^{a} ± 0.02	17.42^{b} ± 0.03

Samples carrying the same superscripts in the same column are not significantly different at [p>0.05]. Values are means of triplicate±SD. **A:** *Pleurotus ostreatus* cultivated on *Pycnanthus ongoleubis*, **B**: *Pleurotu sostreatus* cultivated on *Ceiba pentandra*, **C**: *Pleurotus ostreatus* cultivated on *Cananium* sp.

Amino acid composition of *Pleurotus ostreatus* cultivated on different woody substrates is shown on Table 2. The most abundant amino acids are glutamic acid, arginine, aspartic acid, threonine, leucine and alanine. Chirinang and Intarapichet (2009) had earlier reported that these amino acids are more abundant in *P. ostreatus* and *P. sajor-caju*. However, glutamic acid (9.01g/100g to 10.3 g/100g) was found to be more abundant in *P. ostreatus* cultivated on woody substrate when compared with 5.01 g/100g reported by Chirinang and Intarapichet (2009) for *P. ostreatus* cultivated in Thailand. The possible cause of the differences found in the amino acid composition of this mushroom may be as a result of genetic variation and cultivation process applied in commercial practices. Mendez *et al*. (2005) had earlier reported that genetic variation and cultivation process has a great influence on the nutritional composition of *P.* species. *P. ostreatus* cultivated on *Pycnanthus ongoleubis* had higher and significantly different (P≤0.05) values in all the amino acids when compared with *P. ostreatus* cultivated on t he other woody substrates except Phenlyalanine.

Table 2. Amino acid content of *Pleurotus ostreatus* cultivated on different woody substrates

Amino acid content (g/100g)	A	B	C
Alanine	3.75^{c} ±0.01	3.59^{b} ±0.01	3.55^{a} ±0.01
Arginine	6.30c ±0.01	5.79b ±0.01	5.02a±0.02
Aspartic acid	4.30^{c} ±0.01	4.08^{b} ±0.01	3.80^{a}±0.01
Cystine	0.53^{b} ±0.01	0.53^{b} ±0.01	0.46^{a}±0.01
Glutamic acid	10.2^{c} ±0.01	9.91^{b} ±0.01	9.07^{a}±0.01
Glycine	1.65^{c} ±0.01	1.56^{b} ±0.01	0.43^{a}±0.01
Histidine*	1.10^{c} ±0.00	1.10^{b} ±0.01	1.03^{a}±0.01
Isoleucine*	1.26^{c} ±0.12	1.19^{b} ±0.01	1.10^{a}±0.01
Leucine*	2.31^{c} ±0.01	2.17^{b} ±0.00	1.76^{a}±0.01
Lysine*	1.50^{c} ±0.01	1.42^{b} ±0.01	1.40^{a}±0.01
Methionine*	0.53^{c} ±0.01	0.42^{b} ±0.01	0.47^{a}±0.01
Phenylalanine*	1.27^{a} ±0.01	1.44^{b} ±0.00	1.27^{a}±0.01
Proline	0.433^{b} ±0.01	0.42^{a} ±0.01	0.42^{a}±0.01
Serine	2.10^{c} ±0.01	2.05^{b} ±0.00	1.94^{a}±0.00
Threonine*	2.30^{c} ±0.01	2.25^{b} ±0.01	2.08^{a}±0.01
Tyrosine	1.13^{c} ±0.01	0.97^{b} ±0.01	0.81^{a}±0.01
Valine*	1.63^{c} ±0.01	1.60^{b}±0.01	1.42^{a}±0.01
Total amino acid	42.30	40.56	35.97

Samples carrying the same superscripts in the same column are not significantly different at [p>0.05]. Values are means of triplicate±SD. **A:** *Pleurotus ostreatus* cultivated on *Pycnanthus ongoleubis*, **B**: *Pleurotus ostreatus* cultivated on *Ceiba pentandra*, **C**: *Pleurotus ostreatus* cultivated on *Cananium* sp.

In conclusion, this study established the effect of tropical wood substrates on the mineral and proximate composition of *P. ostreatus*. The results revealed that sawdust from *Pycnanthus ongoleubis* showed good potential as substrate for cultivation based on higher and significantly different (P≤0.05) mineral and proximate contents found in *P. ostreatus* cultivated on it. Sawdust from *Pycnanthus ongoleubis* which most sawmill dispose into the environment could be a good substrate for commercial cultivation of *P. ostreatus*.

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Effect of Ethanol Extract of *Calotropis procera* Root Bark on Carbon Tetrachloride-Induced Hepatonephrotoxicity in Female Rats

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Abstract

Calotropis procera root is used in traditional medical practice for the treatment of various ailments. The possible hepatoprotective and nephroprotective activities of the ethanolic extract of *C. procera* root in female rats were investigated. Carbon tetrachloride (CCl_4) was used to induce hepatotoxicity and nephrotoxicity with significant ($P < 0.05$) increase in the level of serum enzyme markers of hepatotoxicity and nonenzyme markers of nephrotoxicity. Administration of 150 and 300 mg/kg body weight (bw) of the ethanolic extract of *C. procera* root did not protect the liver and kidney from CCl_4-induced toxicity. Pretreatment with the extract rather potentiated the toxicity induced by CCl_4. This research result did not support the reported hepatoprotective activity of the extract. It is advised strongly that caution should be taken when ingesting alcoholic preparations of *C. procera* root.

Keywords: *Calotropis procera*, Hepatonephroprotective, Nephrotoxicity, Hepatoprotective And CCL_4

1. Introduction

Since ancient times, plants, animals and mineral sources have been used to cure and prevent diseases. Plants are the most researched and have been the major source of drugs that are used in modern medicine today and will continue to provide cure for man (Halilu *et al*., 2012). Numerous medicinal plants justify the medicinal uses of herbs (Dahiru and Obidoa, 2009; Sankaran *et al.,* 2010). Hence, the efficacy of the drugs should be tested by standard experimental methods and there should be adequate data from studies to validate the therapeutic potentials (Girish *et al*., 2009).

Calotropis procera belongs to the family Asclepiadaceae and is a soft wooded, evergreen perennial shrub having few stems, few branches and relatively few leaves concentrated near the growing tip. A copious white sap (referred to as the latex) flows whenever the stems or leaves are cut (Ahmed *et al.,* 2005; Liogier, 1995; Sharma *et al.,* 1997; Howard, 1989). *C. procera* is often found growing in open habitat with little competition and it also grows favourably in dry habitat (Parrotta, 2001). *C. procera* is known by various names like Dead Sea apple, Sodom apple, Swallow wort and Milkweed (Gupta *et al*., 2012).

The capsulated root bark powder is effective in diarrhoea (Ahmad *et al*., 2005) and asthma (Singh *et al.,* 1980). The previous pharmacological studies include report of anticancer (Ahmad *et al.,* 2005), antifungal (Hassan *et al*., 2006) and insecticidal activity (Ahmad *et al*., 2006), antifertility and antiulcer activities (Kamath, 2002), hepatoprotective activity against CCl4-induced hepato-oxidative stress in rats (Patil *et al.,* 2011) and antihelmentic activity (Zaman *et al.,* 2012). The reported hepatoprotective activity of the methanolic root extract prompted us to investigate whether the ethanolic root extract of *C. procera* will also exhibit hepatoprotective and if possible nephroprotective activities in CCl_4-induced hepato-nephrotoxicity.

2. Materials and Methods

2.1. Plant Material

Calotropis procera root was collected from McBride ward Jalingo Taraba State, Nigeria in the month of January, 2011. The plant was identified and authenticated

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by the Department of Plant Science, Modibbo Adama University of Technology, Yola. The root bark was pealed and air dried under room temperature (32 ± 2^0C). The dried root bark was powdered and kept in an air tight container until use.

2.2. Animals

A total number of twenty five female Wister strain albino rats weighing between 120 – 130 g were purchased from the National Veterinary Research Institute Vom, Jos, Plateau State. The primary reason for using the female rats was availability at the time of the study. The secondary reason was the unique effect of the root bark on female as abortifacient. The root is used in formulating local abortifacients taken by women. The animals were housed in stainless steel cages in a well ventilated room and were fed with standard pelleted feed (Vital feed, Grand Cereal and Oil Mills, Ltd Jos) and water *ad-libitum* prior to the experiment.

2.3. Preparation of Extract

The root back powder 50 g was placed in a 1000 ml capacity conical flask and 500 ml of ethanol added to it. The mixture was gently stirred, tightly covered and left for 24 hours. It was then filtered using Whatman number 3 filter paper. The filtrate was then placed in a rotary evaporator at 40°C to remove the solvent. The remaining solvent was finally removed using a decitor. The stock solution of the bark extract was prepared by dissolving 1 g of the extract in small quantity of distilled water and later made up to 100 ml with distilled water.

2.4. Experimental Design

Twenty five female rats were divided into five group of 5 animals each (Groups I – V). Treatment was done as follows:

Group I: Normal control, received food and water only.

Group II: Experimental control, received 0.8 ml/kg bw of CCl_4 (1:1 in olive oil) orally.

Group III: Pretreated with 150 mg/kg bw of *C. procera* bark extract for 7 days and 0.8 ml/kg bw of CCl_4 (1:1 in olive oil) orally on the 7th day.

Group IV: Pretreated with 300 mg/kg of *C. procera* bark extract for 7 days and 0.8 ml/kg bw of CCl_4 (1:1 in olive oil) orally on the 7th day.

Group V: Extract control, received 300 mg/kg bw of *C. procera* extract for 7 days and olive oil only. In all groups, both extracts and CCl_4 were administered to rats orally using gastric tube.

The animals were anaesthetized using ether on the 9th day (48 hours after administering CCl_4). Blood was collected into a sterile centrifuge tube by puncturing the retro-orbital plexus. The blood was allowed to clot for 30 min and later centrifuged at 2500 rpm for 15 min to obtain the serum. The serum was later used to assay for the following biochemical parameters:

Alanine aminotransferase (ALT), aspartate amino-transferase (AST), cholesterol (CHOL), triglyceride (TG), total bilirubin (TB), total protein (TP), creatinine and urea. All parameters were analysed using Randox diagnostic kits (Randox Ransod Ltd, UK).

2.5. Statistical Analysis

The results obtained are presented as mean ± SD. Statistical analysis was performed by one-way Analysis of Variance (ANOVA). Individual comparisons between group means were further determined using t-test and considered significant at $P < 0.05$. Software SPSS version 13 was used for the statistical analysis.

3. Results

Results indicate that administration of CCl_4 significantly ($P < 0.05$) increased the serum levels of ALT, AST, TG and total bilirubin while significantly decreasing the serum protein level when compared to normal control. Pretreatment of Groups III and IV with 150 and 300 mg/kg bw respectively of the extract did not protect the liver against the toxicity of CCl_4. Pretreatment with the extract at both doses (Groups III and IV) significantly ($P<0.05$) increased AST serum level in a dose dependent manner with 300 mg/kg bw of the extract increasing the serum ALT levels when compared with group II (CCl_4 only). Pretreatment of rats with 300 mg/kg bw of the extract prior to administration of CCl_4 produced significant ($P < 0.05$) decrease in total protein concentration compared to normal control (Group I) and experimental control (CCl_4 only). Animals administered 300 mg/kg bw of the extract only (Group V) presented significant ($P < 0.05$) increase in serum ALT, AST and TB levels with significant ($P < 0.05$) decrease in serum TG and TP values when compared with normal control (Table 1).

Table 1. Effect of ethanol root bark extract of *C. procera* on markers of CCl_4-induced hepatotoxicity in female rats. Results are mean ± SD for 5 determinations.

GROUP	ALT (U/L)	AST (U/L)	CHOL (mg/dl)	TG (mg/dl)	TB (mg/dl)	TP (mg/dl)
Group I	11.3 ± 1.7	34.7 ± 4.1	83.2 ± 4.8	75.0 ± 1 2.7	0.19 ± 0.02	8.9 ± 0.8
Group II	30.0 ± 6.7^{*}	130.3 ± 8.7^{*}	89.7 ± 7.7	92.6 ± 7.7^{*}	2.45 ± 0.03^{*}	6.1 ± 0.7^{**}
Group III	31.3 ± 3.6	150.0 ± 8.5^{a}	92.3 ± 16.2	89.7 ± 4.7	2.21 ± 0.17^{b}	5.9 ± 0.8
Group IV	63.7 ± $5.9^{a\&}$	192.7 ± $12.8^{a\&}$	80.2 ± 5.6	94.4 ± 6.5	2.39 ± 0.11	3.1 ± 0.5^{bde}
Group V	31.8 ± 6.3^{*}	94.7 ± 8.2^{*bc}	75.1 ± 7.8	44.2 ± 3.0^{bc}	1.91 ± 0.14	3.0 ± 0.3^{b}

* Significantly ($p < 0.05$) higher when compared to Group I
a Significantly ($p < 0.05$) higher when compared to Group II
b Significantly ($p < 0.05$) lower when compared to Group II
c Significantly ($p < 0.05$) higher when compared to Group III and Group IV
& Significantly ($p < 0.05$) higher when compared to Group III
d Significantly ($p < 0.05$) lower when compared to Group III
e Significantly ($p < 0.05$) lower when compared to Group I

Administration of CCl_4 to rats was seen to significantly ($P < 0.05$) increase the levels of biochemical parameters (creatinine and urea) in serum used to evaluate kidney functions. Pretreatment of animals with both doses of extract did not protect the kidney but rather acted synergistically with CCl_4 to significantly ($P < 0.05$)

increase both levels of serum creatinine and urea. Administration of the extract alone at 300 mg/kg bw also significantly increased the levels of the two parameters (Table 2).

Table 2. Effect of ethanol root bark extract of *C. procera* on markers of CCl_4-induced nephrotoxicity in female rats

Treatment	Creatinine (mg/dl)	Urea (mg/dl)
Group I	48.9 ± 5.2	26.6 ± 0.4
Group II	64.2 ± 4.6*	32.8 ± 5.5*
Group III	83.3 ± 3.0*a	52.0 ± 0.1*a
Group IV	81.2 ± 7.1*a	48.0 ± 4.9*a
Group V	63.7 ± 6.0*	36.8 ± 5.0*

Results are mean ± SD for 5 determinations.

* Significantly ($p < 0.05$) higher when compared to Group I

[a] Significantly ($p < 0.05$) higher when compared to Group II

4. Discussion

Carbon tetrachloride induced hepatic injuries are commonly used animal models for the screening of hepatoprotective plant extracts and the magnitude of hepatic damage is assessed by measuring the level of released cytosolic transaminases including ALT and AST in circulation (Agarwal *et al*., 2006). It is established that hepatotoxicity induced by CCl_4 depends on the cleavage of the carbon-chlorine bond to generate trichloromethyl free radical ($CCl_3^{\cdot}$) that reacts rapidly with oxygen to form a trichloromethyl peroxy radical [$CCl_3O_2^{\cdot}$] (Gutierrez and Solis, 2009). This metabolite possibly attack membrane polyunsaturated fatty acids thereby causing lipid peroxidation leading to impairment of membrane function and liver injury (Gonzalez *et al*., 1995). Increased serum levels of both ALT and AST in this study confirm the destruction of the hepatocytes thereby allowing these enzymes to leak into the circulation. The decreased serum level of total proteins in the serum of carbon tetrachloride-treated rats further suggests the severity of liver toxicity. The liver is a major organ of protein synthesis and any disease in the liver can cause damage of hepatocytes with changes in protein and free amino acid metabolism leading to decreases synthesis and increase wasting via catabolism (Yousef *et al.*, 2006; Wallace, 2007 and El- Shafey *et al.*, 2011).

Administration of the extract at both doses of 150 and 300 mg/kg bw did not protect the liver and kidney of rats in this study rather it increased the severity of the damage. This could possibly be due to the toxic effect of the extract itself on the liver and kidney as evidenced by elevated levels of markers of hepato-nephrotoxicity due to the accumulation of toxic doses of the active principles of the root bark extract of the plant. Findings from this study corroborate the results of Pouokam, *et al.* (2011) who reported marked increases in these parameters after administering aqueous extract of *C. procera*. It also supports the results of Buraimoh *et al*. (2011) who showed that the ethanolic leaf extract of *C. procera* is detrimental to the integrity of the liver tissues as evident in the necrotic nature of the liver. Mohammed *et al*. (2012) also reported the toxicity of the root extract in both female and male rats after long exposure. This study contradicts the reported hepatoprotective activity of the root back extract (Basu *et al*., 1992; and Patil *et al*., 2011). This could possibly be due to different medium of extraction and the sex of the animals used. It is strongly suggested that in-depth toxicological study be carried out to eliminate the ambiguity of reports on the extract of the plant. Caution should be taken while ingesting alcoholic preparations of the root of *C. procera* for any medicinal purpose.

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Ameliorative Effect of the Aqueous Extract of *Zingiber officinale* on the Cadmium-Induced Liver and Kidney Injury in Females Rats

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Abstract

The present study was carried out to determine the protective effect of the orally administered aqueous ginger extract (*Zingiber officinale*) against hepatotoxicity and nephrotoxicity induced by high dose of cadmium bromide in adult female albino rats (*Rattus norvegicus*). A total of twenty rats were used in the study. The animals were divided into four groups: Control rats received tap water, second group received cadmium as cadmium bromide at a dose (100 mg Cd^{+2}), third group received Cd^{+2} plus 2g/L aqueous ginger extract, while the last group received only 2g/L ginger extract for 40 days. The result analysis showed that there were several histological changes in the liver and kidney tissue of cadmium treated rats in comparison to control. The cadmium bromide caused degeneration of hepatocytes, inflammatory infiltrated leucocytes and dilation of blood sinusoid lumens in treated rat liver. In kidney, dilatation of tubules, congestion of blood vessels with RBCs and inflammation in cortex of cadmium treated rats were also observed. After administration of aqueous ginger extract to the cadmium treated group, the liver and kidney have approximately returned to the normal histological features. In conclusion, the aqueous extract of *Z. officinale* showed an ameliorative effect against cadmium bromide induced hepatotoxicity and nephrotoxicity.

Keywords: Ginger; Cadmium; Liver; kidney, *Zingiber officinale*.

1. Introduction

Cadmium (Cd) is one of the most toxic heavy metals. This metal is a s erious environmental and occupational contaminant and may represent a serious health hazard to humans and other animals (Kikuzaki and Nakatani 1993 and Mustafa *et al.,* 1993). Several studies have demonstrated the effect of cadmium (Cd) on various organ-systems in the body. It has been reported that Cd induced nephrotoxicity, testicular damage, lung damage, hepatotoxicity, and body weight loss (Ige *et al.*, 2011). Absorption of ingested Cd is only about 5% and after absorption it accumulates in the liver and then in the kidney (Smalinskienel *et al.,* 2006) and its half-life is very long, exceeding 10 yr (Kramarova *et al*., 2005).Cadmium can accumulates and cause a n umber of lesions in the body tissues, such as the liver, kidney and testis (Egwurugwu *et al.*, 2007, Stoilova *et al.*, 2007). Liver of rats treated with $CdCl_2$ showed that there were degenerative changes in numerous hepatocytes; the cells were enlarged and had light and foamy cytoplasm filled with numerous vacuole-like spaces and in the kidney showed that there were many areas of tubular damages were observed in all treatment animals. These renal damages appeared as hypertrophy and degeneration of epithelia of renal tubules with distinct of mononuclear cells infiltration. The walls of the blood sinusoids were dilated and showed numerous Kupffer cells (Mahran *et al.*, 2011). The toxic effects of cadmium on or ganisms include nephrotoxicity, carcinoegenicity, hepatotoxicity and endocrine disruption (Serafim and Bebianno, 2007).

Ginger (*Zingiber officinale*) is one of the world's best known spices, and it has also been universally used throughout history for its health benefits. The main constituents of ginger include volatile oil, phenolic derivatives (zingerone) and oleoresin (gingerols and shogaols) are main antioxidant compound in ginger (Kikuzaki and Nakatani, 1993). Ginger extract possesses antioxidative characteristics, since it can scavenge superoxide anion and hydroxyl radicals. *Z. officinale* was found to inhibit the activity of lipoxygenase and peroxidation (Topic *et al.*, 2002). Barakat and Mohamed (2011) showed ability of ginger to protect the liver against

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the oxidative stress and hepatocellular injury that follows a supra therapeutic dose of acetaminophen.

The aim of the present study was to evaluate the effect of ginger water extract in protecting against the toxic effect of cadmium bromide on kidney and liver of rats.

2. Materials and Methods

2.1. Animals and Housing

The study was carried out on twenty adult female Wistar albino rats (*Rattus norvegicus*). All rats were weighing about (271.55 ± 8.3738 gm) and (10) weeks of age at the time the experiment was started.

The animals were bred and housed in plastic cages (56 x 39 x 19 cm) bedded with wooden chips. The animals were purchased from the Animal House of the Department of Biology, College of Science, Salahaddin University-Erbil. Climate controlled conditions were maintained and temperature was set as (22± 2^{o}C). Regular 12-hours diurnal cycles were kept using an automated light-switching devise, The rats were given standard laboratory chow containing 0.5% NaCl, 22% protein and 4-6 dietary fat and allowed drinking water *ad libitum*.

2.2. Experimental Design

Albino rats used in this experiment (were) grouped into the following groups:

2.3. Control Group

The rats of this group received standard rat's diet containing 0.5% NaCl, 22% protein and 4-6% dietary fat and tap water *ad libitum* (Krinke 2002).

Group 1: Cadmium bromide solution

The rats of this group received Cadmium bromide solution daily in the drinking water 100 mg (Cd^{+2}) for 40 days (Benoff *et al.*, 2008).

Group 2: Cadmium bromide & aqueous Ginger extract.

The rats of this group received Cadmium bromide (100 mg) and ginger (2 g/L) daily in the drinking water for 40 days (Hasanabad *et al.*, 2005).

Group 3: Dose of aqueous Ginger extract.

The rats of this group received ginger daily in the drinking water (2 g/L) for 40 days.

2.4. Collection of Liver and Kidney Samples

At the end of the experiment, the rats after being starved over night were anesthetized with ketamine hydrochloride (100mg/Kg) and then decapitated. Liver and kidney isolated after dissecting the animals into tubes with fixatives (chromaid), allowed to fix for 24 hours and then stored at 70% ethanol for histological analysis. The tissues were processed and embedded in paraffin wax. Thick sections(6µm) were obtained and stained by hematoxylin and eosin (H&E) and examined under light microscope to determine the morphological changes.

3. Results

The present investigation showed several histological alterations in the liver and kidney of cadmium treated rats in comparison to control (Figure 1-3). As shown in Figure 1b and c, cadmium bromide caused degeneration of liver cells especially hepatocytes, in which these cells were seen shrunken with condensed nuclei. Inflammatory infiltrated leucocytes forming foci were detected around blood vessels (Figure 1b). Blood sinusoid lumens were seen dilated in most regions of cadmium treated rat liver (Figure 1c). After given ginger to the cadmium treated group, the liver tissue restored most of its normal histological features (Figure 1d).

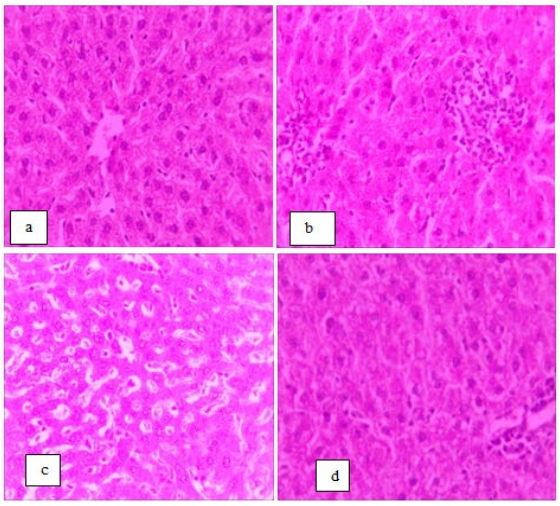

Figure1. Sections through liver, A)control showing normal structure of liver,400X,H&E,B) cadmium treated rat liver showing inflammatory leucocytes infiltration near the blood vessel, 400X, H&E, C) cadmium treated rat liver showing blood sinusoid dilatation, 100X, H&E, D) cadmium plus ginger showing approximately normal structure, 400X,H&E.

As shown in Figure 2a and b, the kidney of control group showed normal histological structure, while when cadmium b romide was given to the rats, it caused dilatation of kidney tubules, congestion of blood vessels with RBCs (Figure 2c) and inflammation in certain regions of the kidney cortex (Figure 2d). When ginger was administered in combination consequence with cadmium bromide, it has caused attenuation of the nephrotoxicity caused by cadmium (Figure 3).

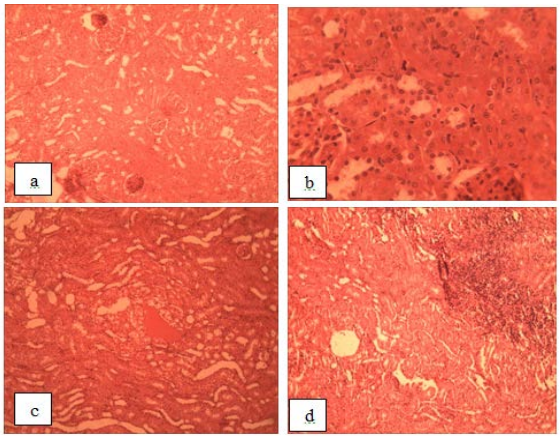

Figure 2. Sections through kidney, A) & B) control showing normal structure of kidney, 100X & 400X respectively, H&E, C) cadmium treated rat kidney showing dilatation of kidney tubule and congested blood vessel,400X,H&E, D) cadmium treated rat kidney showing Inflammatory foci in the cortical region, 100X H&E.

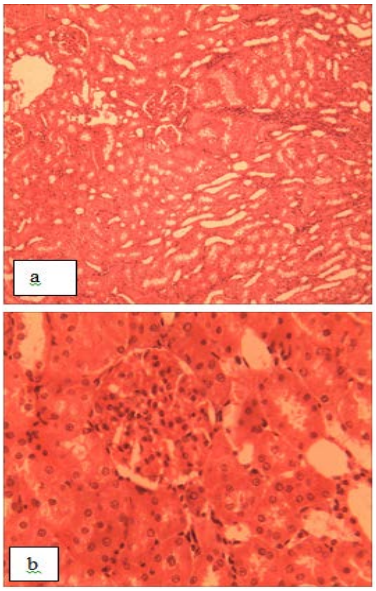

Figure 3. Sections through kidney of cadmium plus ginger treated rats showing approximately normal structure of kidney, A) 100X b) 400X.

4. Discussion

Histopathological studies revealed that alterations occurred in the hepatic and kidney architecture of cd treated rats. The kidney has been recognized as a critical target organ of Cd toxicity. Cd-treated rats showed severe cellular degeneration, necrosis, and hepatocytes and localized fatty degenerations. This agrees with earlier studies (Ige *et al.,* 2011; Mahran *et al.*, 2011). They showed that there were changes in liver including, a blurred trabecular structure, vacuolar degeneration and increased density of nuclear chromatin with very compact nuclear structure of hepatocytes. Moreover; mononuclear cell infiltrations and necrosis of single cells were also observed. These morphological hepatic changes may be due to the decline activities of hepatic Superoxide dismutases (SOD) and an increase hepatic Malondialdehyde (MDA**)** seen in Cd-treated rats (Mohammad 2011).

These changes may also be due to direct toxic effects of the toxicants on hepatocytes since the liver is the site of detoxification of all types of toxins and chemicals (Soufy *et al.*, 2007). The liver is the organ most associated with the detoxification and biotransformation of foreign compounds that enter the body. However, its regulating mechanism can be impaired by accumulated toxicants which could result in structural damage (Camargo and Martinez, 2006). Furthermore, the liver is one of the critical target organs after chronic exposure to cadmium (Sobha 2007). The liver accumulates substantial amounts of cadmium after both chronic exposures.

In kidney tubules, degeneration and hypertrophy of epithelial cells and dilation of glomeruli and massive local haemorrhage of the renal tissues were observed (Obianime and Roberts 2009). The mechanism of Cd-induced kidney damage is considered to be related to increased oxidative status. Increasing of oxygen of free radicals production seem to be induced by the interaction of Cd with mitochondrial structure (Tang and Shaikh 2001).

In ginger co-treated animals, we noticed an improvement in the Cd-induced damage in the liver and kidney. Our results were in agreement with published data by Gehan and Ayman (2010) who observed that ginger expressed an antagonistic action on cadmium toxicity. This protective effect could be attributed to the fact that ginger contains high content of antioxidants that makes it a free radical scavenger (Krishnakanta and Lockesh, 1 993). Moreover, another study by Yuki Masuda *et al.* (2008) reported that the antioxidant activity of ginger might be due to not only radical scavenging activity of antioxidants but also their affinity to the substrates. Egwurugwu et *al.* (2007) reported that ginger therapy was more effective as more Cd intake was avoided. We assumed that ginger through the said antioxidant activities, first improved the blood balance with the confirmed results improving the liver functions followed by improving the kidney functions. This is the first hypothesis explaining the role of ginger in improving liver and kidney functions. Finally, our results demonstrated the ameliorative effect of aqueous ginger extract (*Zingiber officinale*) administration on the Cd induced toxic structural changes in the liver and kidney tissues of the rats. These results validate the hypothesis that the metabolism and toxic action of Cd may be modulated by aqueous ginger extract supplementation.

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Prevalence of Congenital Red-Green Color Vision Defects among Various Ethnic Groups of Students in Erbil City

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Abstract

The incidence of color blindness varies from race to race and in different geographical area. Since, there is no such report about the prevalence of color blindness in the Kurdistan region (North of Iraq), the present study had been conducted to find out the incidence of color blindness among preliminary, secondary and college students of Erbil city, Kurdistan region – Iraq. Participant's (n=1856, 1275 males, 581 females, age group 7 to 25 years) color vision was tested by using Ishihara chart. Among 1275 male students, 108 students (8.47%) were found to be color blind: 42 of them showed deuteranomalia, 39 protanomalia, 20 deuteranopia and 7 protanopia. Among 581 female students, 8 females (1.37%) were found to be color blind: 5 of them showed protanomalia, 2 deuteranomalia and 1 deuteranopia. Data observed by Chi-square test showed there was no significant difference between ethnic groups of both male and female students. Studies on color blindness in Kurdistan region are very few; we hope this student-based investigation is meant to fill a gap in this field.

Keywords: Color Vision Deficiency, Ishihara Color Test, Ethnic Groups, Erbil City.

1. Introduction

Color blindness is an abnormal condition characterized by the inability to clearly distinguish different colors of spectrum. Human color vision is normally trichromatic i.e. the mixture of red, green, and blue lights (Curcio *et al.*, 1990). Most color vision defects are congenital and permanent. Red-Green defects (Protan and Deutan) show the highest prevalence in the general population (Citrik *et al.*, 2005). Impaired color vision, in the case of red-green color blindness, is genetically determined by X-linked recessive inheritance and thus occurs in males but is transmitted via female and about 8.0% of all women are carrier of it (Guyton and Hall, 2005). The genes responsible for red-green Color vision deficiency are located on the long arm of the X-chromosome within the Xq28 band (Deeb and Kohl, 2003; Filosa *et al.*, 1993; Norn, 1997), while the blue pigment gene resides on the 7th chromosome (Deeb and Kohl, 2003; Deeb, 2004; Motulsky, 1988). Being a genetic disorder, the incidence, of color blindness, varies from race to race and different in different geographical regions of the world inhabited by people of different ethnicity (Rahman *et al.*, 1998). John Dalton was the first scientist to give a clear description of his affliction of color blindness in 1798 (Sutender, 1995).

Color blindness is one of the extensively studied genetic marker in the study of human variation and it is an important genetic trait in the field of human genetics. It has been suggested that natural selection operates in higher intensity color vision deficiency among many primitive populations (Pickford, 1963; Roberts, 1967).

classification of the color vision deficiency

Protanomaly	Red weakness
Deuteranomaly	Green weakness
Tritanomaly	Blue weakness
Protanopia	Red deficiency
Deuteranopia	Green deficiency
Tritanopia	Blue deficiency
Achromatopia	Absolute color blindness

In a normal trichromat, three wavelengths are required to match wavelength. Dichromacy occurs when there are only two cones functioning. Monochromats and achromats only need one wavelength to match the reference color. A mild color deficiency is present when one or more of the three cones function "poorly". A more sever color deficiency is present when one of the cones does not function at "all" or is missing. Red green

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deficiency is by far the most common form of color blindness. The scientific basis for the same is that, DNA sequences of the red and green receptor gene are so similar, that it is easy for mistakes to occur during the development of egg and sperm, as genetic material is replicated and exchange between chromosomes. Those with a less common type have difficulty distinguishing blue and yellow. In very few cases, color deficiency exists to an extent that no colors can be detected; only shades of black, white gray are seen (Tortora and Grabowski, 1996; Foster, 1991).

The incidence of color blindness has been reported from various populations of several countries. There is no report of such study undertaken about the prevalence of color blindness of the Kurdistan Region of Iraq. So the present study had been conducted to find out the incidence of color blindness and racial difference of it among the students of Erbil city, Kurdistan region, North of Iraq.

2. Materials and Methods

This study was carried out among students of some preliminary and secondary schools (Fenk, Ishk, Fatema Zahra, Ankawa, Bablian and Halgurd) and also colleges of University of Salahaddin in Erbil city. From these schools and colleges total number (1856) individuals: (1275) males and (581) females with the age (7-25) and belonging to various ethnic groups: 1090 Kurdish, 269 Arabic, 240 Turkman and 260 Kldan were tested for color vision deficiency.

This study was done with the help of Ishihara chart. This chart consists of polychromatic plates containing printed figures made up of colored spots on a background of similarly shaped colored spots. The figures are intentionally made up of colors that are liable to look the same as the background, to an individual who is color-deficient. The color vision testing plates are held at (75 cm) from the student and tilted at right angle to the line of vision. The tests were performed binocularly in sufficient indirect daylight as recommended by Ishihara. The color Ishihara chart was shown to all participants and they were asked to read the impressions in the color chart. The impression perceived by a person with normal color vision was different from the impression perceived by a person with color vision deficiency. The types of color blindness were differentiated with the help of key provided with the chart.

3. Results

This study was performed on he althy preliminary, secondary school and college students to determine the prevalence of congenital color blindness in Erbil city.

A total of 1856 participants including 1275 males and 581 females with age range from (7-25) years completed the study.

From the data obtained into Table 1 (8.45%) of Kurdish, (9.44%) of Arabic, (8.52%) of Turkman and (7.40%) of Kldan male students were found to have congenital color vision defective with variation in different types of color blindness: red weakness (protanomaly), green weakness (deuteranomaly), red deficiency (protanopia) and green deficiency (deuteranopia).

The results for ethnic variation among females under study are shown in the Table 2 (1.20%) of Kurdish, (2.24%) of Arabic, (1.56%) of Turkman and (1.05%) of Kldan female students were found to have congenital color defective with variation in different types of color blindness.

The distribution of different types of color blindness among male students of the present study is presented in Figure 1. Among the color blinds, 42, 39, 20 and 7 male students were the victims of Deuteranomaly, Protanomaly, Deuteranopia and Protanopia respectively.

The comparisons between ethnic groups were showed no significant difference using Chi-square test, in both male and female students.

Table 1. The percentage of phenotypic frequency of the different types of color vision deficiency among male students of different ethnic groups

Male							
Ethnic Groups	**No. studied**	**No. defected**	**Defected color vision %**	**Red weakness%**	**Green weakness%**	**Red deficiency%**	**Green deficiency%**
Kurd	757	64	8.45	3.17	3.43	0.52	1.32
Arab	180	17	9.44	3.33	2.77	1.11	2.22
Turkman	176	15	8.52	2.84	3.40	0.56	1.70
Kldan	162	12	7.40	2.46	3.08	0	1.85
Total	1275	108	8.47	3.05	3.29	0.54	1.56

Table 2. The percentage of phenotypic frequency of the different types of color vision deficiency among female students of different ethnic groups

Female							
Ethnic Groups	**No. studied**	**No. defected**	**Defected color vision %**	**Red weakness %**	**Green weakness %**	**Red deficiency%**	**Green deficiency%**
Kurd	333	4	1.20	0.6	0.3	0	0.3
Arab	89	2	2.24	1.12	1.12	0	0
Turkman	64	1	1.56	1.56	0	0	0
Kldan	95	1	1.05	1.05	0	0	0
Total	581	8	1.37	0.86	0.34	0	0.17

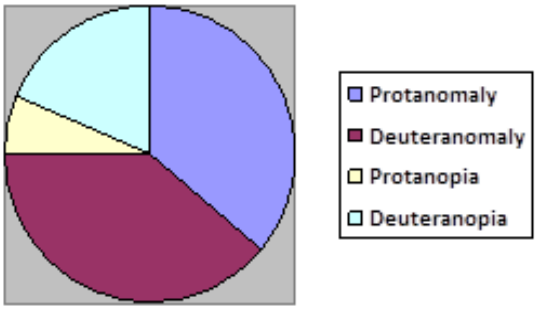

Figure 1. Percentage distribution of different types of color blindness among male students of the present study

4. Disscusion

The percentage distributions of color blindness in the different ethnic groups and countries are found to be variable: in our study the prevalence of color blindness among the male students (8.46%) were found to be similar recorded in America, 8.0% (Mueller and Young, 1995), Tehran, 8.18% (Modarres *et al.*, 1996), Iraq, 8.19% (Al-Amood *et al.*, 1981), Denmark, 8.7% (Norn, 1997), Jordan, 8.72% (Al-Aqtum and Al-Qawasmeh, 2001) and India, 8.73% (Shah *et al*., 2013). However, the prevalence of the color blindness among male students in our present study is higher than that of Pakistan, 1.1% (Alam *et al.*, 2008), India, 1.12% (Dakshayani and Gangadhar, 2006), 2.4% (Kalamma *et al.*, 2008) a nd 2.4% (Luxmi and Kapooe, 2011), China, 3.0% (Huang *et al.*, 1990), Nepal, 3.8 (Niroula and Saha, 2010), Philippine, 5.17% (Cruz *et al.*, 2010) and Malaysia, 6.7% (Balasundaram and Reddy, 2006).

In the present study the prevalence of color blindness among female students (1.37%) were found to be similar with some researches done in Saudi Arabia 0.75% (Oriowo and Alotaibi, 2008), Aligarh and Simla 0.8% (Rahman *et al.*, 1998), India 1.04% (Mehra, 1963), Punjab 1.1% (Bansal, 1967) and India, 1.69% (Shah *et al*., 2013).

Since the color blindness is genetically transmitted, its distribution is likely to be variable in different ethnic groups. The percentage distributions of color blindness in our study were found different in different ethnic groups: highest in Arab male students (9.44%) and female students (2.24%) these results are similarly recorded by Al-Amood *et al* in 1981 among Arabs of Iraq. The higher prevalence rate of color blindness may be due to the hidden effect of consanguineous marriages.

Although several therapies have been proposed (e.g. electrical eye stimulation, Iodine injections and large doses of vitamins), there are no t reatments or surgical procedures to improve the quality of an individual's chromatic vision (Richer and Adams, 1984).

Detection of color blindness at an early age can be extremely useful to avert or prevent certain occupational hazards, including death in the case of drivers of locomotives and automobiles.

In order to find out the exact incidence of color blindness among the different ethnic groups in Kurdistan region, a further study with a large sample is necessary.

5. Conclusion

This survey should serve as a call for other researchers to carry out more work on c olor vision deficiency in Kurdistan and Iraq. Also the information on red-green defection among males and females in Kurdistan has been lacking. The present study found the prevalence rate of (CVD) in Erbil city male students (8.46%) and female students (1.37%).

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Short Communication

Molecular Characterization and Phylogenetic Analysis of Cellulase Producing *Streptomyces griseorubens* (Strain St-1) Isolated from Indian Soil

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Abstract

The present study was aimed at characterization of the cellulose degrading actinomycete strain St-1, isolated from soil samples of Patna region in India, on the basis of morphological, cultural, physiological and molecular characterization. Based on morphological and analysis of 16S rRNA sequence, the strain St-1 was identified as *Streptomyces griseorubens* (Accession no. AB 184139). The strain was able to grow in a wide range of pH (5-11) and temperature (4-45 ^{0}C) with 7 and 28^{o}C being the optimum pH and temperature, respectively. The strain was able to survive at up to 6 % NaCl concentration through the optimum growth was observed at 1% NaCl concentration.

Keywords: Cellulase Producing Actinomycete, *Streptomyces griseorubens*, 16S rRNA Analysis

1. Introduction

Actinomycetes, phylogenetically defined as a number of taxa within the high G+C subdivision of the gram-positive phylum (Embley and Stackebrandt, 1994), are involved in important processes in a wide range of habitats (Mincer *et al*., 2002). Among actinomycetes, *Streptomyces* are quite significant as they bring about the decomposition of various plant based polysaccharides by the production of wood hydrolytic enzymes, such as cellulases and hemicellulases (Crawford and Sutherland, 1979). Cellulases provide a key opportunity for achieving tremendous benefits of biomass utilization (Wen *et al*., 2005) for the possible use in large scale biorefining. Glucose, from appropriate hydrolysis of this cellulosic biomass, under the treatment of advanced biotechnology can be used in different applications such as production of fuel ethanol, single cell protein, feed stock, industrially important chemicals and so on (Lynd *et al*., 2002). The objective of this study was to describe the morphological, biochemical, physiological, cultural and the molecular characteristics of the cellulase producing *Streptomyces griseorubens* Strain St-1 isolated from soil samples collected from Patna region in India

2. Materials and Methods

2.1. Isolation of Microorganism

Cellulase producing strain St-1, displaying the greatest cellulase activity on CMC agar (carboxymethylcellulose 0.5 g/l, $NaNO_3$ 0.1 g/l, K_2HPO_4 0.1g/l, $MgSO_4$ 0.05g/l, yeast extract 0.05g/l, agar 15 g/l) (Kasana *et al*., 2008) plates using Congo red test, was selected among the cellulolytic strains isolated from soil samples collected from Patna region on Starch Casein Agar medium (Soluble starch 10 g/l, Casein 0.3 g/l, K_2HPO_4 2 g/l, $CaCO_3$ 0.02 g/l, $FeSO_4 7H_2O$ 0.01 g/l, KNO_3 2 g/l, $MgSO_4 7H_2O$ 0.05 g/l, NaCl 2 g/l, Agar 18 g/l) by standard serial dilution technique. The strain was then purified by single streak plate technique (Thakur *et al*., 2007) and preserved on s lants of Nutrient Agar at 4^{o}C with periodic sub culturing for further investigation. Colony morphology on S tarch Casein Agar and Nutrient Agar regarding the color of the mycelium, pigmentation and texture of the culture were observed and recorded after an incubation period of 7days at 28 ^{0}C. Micro-morphological studies and photographic observations were done with a l ight microscope (Carl Zeiss Microscope with camera) using cover-slip method in ISP2

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agar (yeast extract 4 g/l, malt extract 10 g/l, glucose 4 g/l, agar, 20 g/l) (Shirling and Gottlieb, 1966) after Gram staining.

2.2. Cultural characteristics

The cultural characteristics were studied by growing the strain on different ISP and non ISP media according to the guidelines of the International Streptomyces Project (Shirling and Gottlieb, 1966). Nine different media, including ISP_1, ISP_2, ISP_3, ISP_4, ISP_5, ISP_6, ISP_7, Starch Casein Agar and Nutrient Agar were selected for describing the culture characteristics.

2.3. Physiological characteristics

Physiological criteria included the ability of the isolate to utilize different carbons as described by Pridham and Gottlieb (1948) using plates containing basal medium ($(NH_4)_2SO_4$, 2.64g; KH_2PO_4, 2.38g; K_2HPO_4, 5.65g; MgSO4, $7H_2O$, 1g; standard saline solution, 1ml; agar, 15g; H_20, 1000 ml, pH 7.2) supplemented separately with 1% dextrose, sucrose, lactose, fructose, mannitol and inositol. The plates were incubated at 28°C and the growth was read after 7 days of incubation. The pH and temperature for growth were determined by growing the strain on NA plates with variable pH values (3, 5, 7, 9 and 11) and temperatures (4°C, 15°C, 26°C, 37°C, 45°C, 50°C, 55°C and 60°C) for 7 days. The growth of strain St-1 on 10 different concentrations of NaCl (1% to 10%) in the medium was studied according to the method of Tresner *et al*., 1968.

2.4. Molecular characterization

2.4.1. Isolation and amplification of chromosomal DNA

The chromosomal DNA of the strain St-1 was isolated as described by Houda *et al*. (2009). Amplification of the 16S rRNA was performed using a PCR kit (Sino-American Biotechnology Co., Beijing). PCR mixture consisted of 1 µl (80 ng) chromosomal DNA, 0.5 µl (200 µM) of each deoxynucleoside triphosphate (New England Biolabs), 0.8 µl (80 ng) Universal primer 27F (5'-AGAGTTTGATCMTGGCTCAG-3'), 0.8 µl (80 ng) Universal primer 1492R (5'-TACGGYTACCTTGTTA CGACTT-3'): *Escherichia coli* 16S rDNA numbering system (Brosius *et al.*, 1978), 0.5 µl (2.5 Units) Taq Polymerase, 2.5 µl of 1X Standard Taq buffer and 18.9 µl autoclaved water to make the final volume of 25 µl. PCR was carried out in a thermocycler (Eppendorf Mastercycler gradient) using PCR program that included initial denaturation at 95°C for 5 min followed by 30 cycles of denaturation at 95°C for 1 min, annealing at 55°C for 1 min and primer extension at 75°C for 2 min.

At the end of the cycles, the mixture was kept at 75°C for 10 min for a final extension.

Approximately 1.5 kb amplified 16S rRNA gene fragment was separated by agarose gel electrophoresis, extracted using Qiaquick gel extraction kit (Qiagen) and purified.

2.4.2. Sequencing of 16S rRNA gene

The amplified and purified PCR product was sequenced by the dideoxy chain terminator method using a Big Dye Terminator kit followed by capillary electrophoresis on an ABI 310 Genetic Analyzer (Applied Biosystem). The primers used for sequencing were 27F, 341F (5'-CTGGGAGGCAGCAGTGGG-3'), 786 F (5'-GATTAGATACCCTGGTAG- 3'), 536 R (5 '-GTATTACCGCGGCTGCTG – 3') and 1492R. The 16S rRNA sequence of the strain was compared with sequences deposited in the NCBI GenBank database and EZTaxon Version 2.1 using BLAST program.

2.5. Phylogenetic analysis

Phylogenetic trees were constructed using the Neighbor-Joining method in MEGA program version 4.1 (Tamure *et al.*, 2004). The 16S rDNA gene sequences of the type strains of the various genera used in this study were retrieved from the GenBank database and used for cladistic analysis. These sequences have been chosen as reference sequences.

Nucleotide sequence accession number:

The 16S rRNA gene sequence of the strain was deposited in GenBank and an accession number (AB184139) was obtained.

3. Results

3.1. Morphological characteristics

The strain was identified as an actinomycete by colony morphology. Morphological observation of the cultures of the strain St-1 on Starch Casein Agar showed luxuriant grayish white aerial mycelium and yellow substrate mycelium while no diffusible pigmentation was observed. Micro-morphological observation revealed Gram positive filamentous structure.

3.2. Cultural characteristics

The cultural characteristics of the strain listed in Table 1 showed different degrees of growth on most media. The strain was able to grow luxuriantly on all media except ISP_2 and ISP_3. Typically, the colony showed white to grey aerial mycelium. The substrate mycelium was colorless on all the media except ISP_1, ISP_6 and Starch Casein Agar on which it was brownish yellow, brown and yellow, respectively. The strain produced a vivid yellow diffusible pigment on ISP_1 media.

Table 1. Cultural characteristics of *S.griseorubens* (strain St-1)

S. No. Name of the medium	*S.griseorubens* (strain St-1)
1. **Tryptone Yeast Extract Agar (ISP_1)**	
Growth	Luxuriant
Aerial mycelium	White
Substrate mycelium	Brownish yellow
Pigmentation	Yellow
2. **Yeast Extract Malt Extract Dextrose Agar (ISP_2)**	
Growth	Poor
Aerial mycelium	White
Substrate mycelium	Colourless
Pigmentation	Nil
3. **Oatmeal Agar (ISP_3)**	
Growth	Poor
Aerial mycelium	White
Substrate mycelium	Colourless
Pigmentation	Nil
4. **Inorganic Salt Starch Agar (ISP_4)**	
Growth	Luxuriant
Aerial mycelium	Grayish white
Substrate mycelium	Colourless
Pigmentation	Nil
5. **Glycerol Asparagine Agar (ISP_5)**	
Growth	Luxuriant
Aerial mycelium	White
Substrate mycelium	Colourless
Pigmentation	Nil
6. **Peptone Yeast Extract Iron Agar (ISP_6)**	
Growth	Luxuriant
Aerial mycelium	White
Substrate mycelium	Brown
Pigmentation	Nil
7. **Tyrosine Agar (ISP_7)**	
Growth	Luxuriant
Aerial mycelium	Light grey
Substrate mycelium	Colourless
Pigmentation	Nil
8. **Starch Casein Agar**	
Growth	Luxuriant
Aerial mycelium	Grayish white
Substrate mycelium	Yellow
Pigmentation	Nil
9. **Nutrient Agar**	
Growth	Luxuriant
Aerial mycelium	White
Substrate mycelium	Colourless
Pigmentation	

3.3. Physiological characteristics

The physiological factors influence the rate of growth of actinomycetes and an essential tool for their classification and identification as reported by Kampfer *et al.* (1991). Six carbon sources were used in our study and the strain St-1 was able to utilize all the tested carbon sources efficiently except dextrose. Growth of the strain St-1 was observed in the pH range of 5-11 with the optimum being 7. The temperature range for growth was 4-45^{0}C and optimum growth was observed between 26 and37^{o}C. The strain showed salt tolerance up to 6% with optimum growth at 1% NaCl. Hence, it can be inferred that the strain is not a halophile. T he physiological characteristics are presented in Table 2.

Table 2. Physiological characteristics of *S.griseorubens* (strain St-1)

Physiological characteristics	Growth of *S. griseorubens* (strain St-1)
Carbon utilization	
Dextrose	++
Sucrose	+++
Lactose	+++
Fructose	+++
Mannitol	+++
Inositol	+++
Range of pH	
3	Nil
5	+++
7	+++
9	+++
11	+++
Range of temperature	
4^{0}C	+
15^{0}C	++
26^{0}C	+++
37^{0}C	+++
45^{0}C	++
50^{0}C	-
55^{0}C	-
60^{0}C	-
NaCl tolerance	
1%	+++
2%	++
3%	++
4%	++
5%	++
6%	++
7%	-
8%	-
9%	-
10%	-

- No growth; + poor growth

3.4. Molecular characterization and Phylogenetic analysis

The phenotypic characteristics showed that the isolate *Streptomyces griseorubens* strain St-1 belongs to the genus *Streptomyces*. The taxonomic position of the isolated strain was confirmed by 16S rRNA gene sequencing (Figure 1). BLAST analysis of 1411 bps of the 16S rRNA gene sequence of strain St-1 in EZTaxon Version 2.1 software revealed 100% similarity with *Streptomyces griseorubens*. The sequences of the first ten hits were retrieved from the database and used to construct a phylogenetic tree by Neighbour-Joining method (Saitou and Nei, 1987) in MEGA Version 5.1 software (Tamura *et al*., 2011**)**. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura *et al*., 2004) in the units of the number of base substitutions per site.

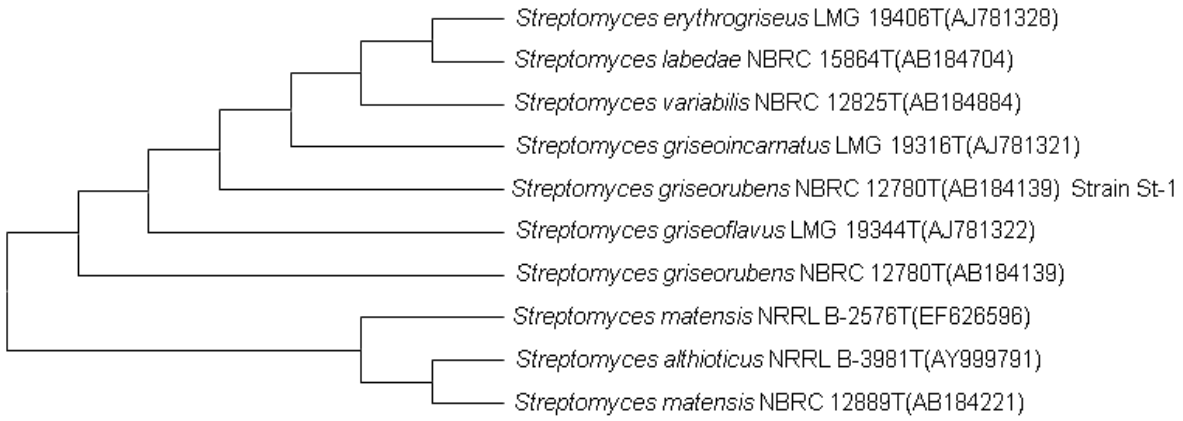

Figure 1. Evolutionary relationships of taxa: The optimal tree with the sum of branch length = 0.60185941 is shown. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree.

4. Conclusion

The present study was aimed at isolation and characterization of the isolated cellulose degrading actinomycete *Streptomyces griseorubens* strain St-1 from soil on the basis of morphological, cultural, physiological and molecular characterization. Growth of the strain at different pH and temperature reveals that the strain possesses high tolerance to the variability of pH and temperature that can be helpful in the survival of this cellulose degrader in the fluctuating growth conditions predominant in nature.

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Case Report

Phenylketonuria in Sohag: A Preliminary Study

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Abstract

Phenylketonuria (PKU) is one of the commonest inborn error of metabolism, it is an autosomal recessive metabolic genetic disorder characterized by a mutation in the gene for the hepatic enzyme phenylalanine hydroxylase (PAH), rendering it nonfunctional. The diagnosis of this disorder can be confirmed by analysis of urine components. The present study aimed to assess the prevalence of PKU among children aged 6 months to 6 years in Sohag governorate Egypt, its relationship to malnutrition and identifying families with higher predisposition to having children with inborn errors of metabolism. One hundred children were selected from 18,000 patients seen in the pediatric neuropsychiatry clinic of Sohag University hospital over three years, between May 2008 - May 2011. They were presented with clinical symptoms suggestive of probable preliminary diagnosis of PKU. Proper clinical and laboratory investigations, including ferric chloride test in urine, total protein and albumin in serum, were screened to confirm the diagnosis. PKU was diagnosed in two children cases. The diagnosed cases were suffering from mild malnutrition represented by low levels of serum albumin and total protein comparable to cases of Marasmus and kwashiorkor or other deficiencies like rickets. Screening of the newborn with special emphasis on PKU is highly recommended before discharge from the nursery for children delivered in the hospital or on first visit to the clinic for children delivered at home. Early detection would help prevent serious and permanent neurological impairment.

Keywords: Phenylketonuria (PKU), Inborn Errors of Metabolism (IEMs), Sohag Governorate, Egypt, Phenylalanine Hydroxylase, Neurological Impairment, Newborn Screening.

1. Introduction

Many inborn errors of metabolism (IEMs) are classified as organic acidemia, in which organic acids accumulate in the urine (Kuhara, 2007). Human urine contains numerous metabolic intermediates at a variety of concentrations that can provide clues for diagnosing inborn errors of metabolism and other genetic mutations (Albers *et al*., 2001; Blau *et al*., 1996; Imamura *et al*., 1999; Lee *et al*., 2011).

For most of the cases with IEMs, the clinical presentations are variable and nonspecific and routine laboratory tests do not usually identify the etiology of the disease. Metabolite analysis can comprehensively detect enzyme dysfunction caused by a variety of abnormalities (Kuhara, 2007; Ponzone *et al.,* 1990).

Phenylketonuria (PKU) is an autosomal recessive genetic disorder characterized by a deficiency in the enzyme phenylalanine hydroxylase. When this enzyme is deficient, phenylalanine accumulates and is converted to phenyl pyruvate, which is also known as phenylketone, which is detectable in the urine. Other unknown factors also interfere in determining the metabolic profile of PKU (Oh *et al*., 2004; Blau *et al*., 1996). It is important to emphasize that early diagnosis and treatment are critical for patients with IEMs. The present study aims to identify the prevalence of PKU among children aged 6 months to 6 years seen in the pediatric clinic of Sohag University. Relationship of this disease to malnutrition was also investigated through determining the total protein and albumin in the sera of the children. Families predisposed to have children with this IEMs are also identified and counseled. It is important to test the validity and the practicality of using inexpensive biochemical laboratory tests beside the clinical neurophysiologic criteria for diagnosing PKU in an underprivileged locality.

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2. Patients and Clinical Examination

The study was approved by the clinical ethics committee of the Sohag Faculty of Medicine. One hundred children, aged 6 months to 6 years, were selected from 18,000 patients examined in the pediatric clinics of Sohag University hospital over 3 years from May 2008 to May 2011. They presented with clinical symptoms suggestive of probable preliminary diagnosis of PKU. Depending on the severity of symptoms they were seen either as outpatients or were admitted as inpatients. These symptoms included delayed milestones of growth, vomiting since birth, blonde hair and eyebrows, jaundice and organomegaly, rickets, diarrhea, pneumonia, convulsions and skeletal deformities. Exclusion criteria from the study included cerebral palsy, myasthenia gravis, primary muscular dystrophies, myotonia congenita, polymyositis, mental retardation, epilepsy, Friedrich's ataxia, hereditary spastic paraplesia, poliomyelitis and cardiostenosis.

For all children participating in the study, informed written consent was obtained from a parent or next of kin. Children and parents were interviewed for full social and clinical history taking, including consanguinity between parents, history of sibling death, congenital anomalies and IEMs , especially PKU. Physical examination of the children covered the general look, hair changes, developmental milestones, head circumference and congenital anomalies. Full neurological examination was carried out for general behavior, muscle power and reflexes. Chest and abdominal examination was done for detection of organomegaly.

2.1 Biochemical methods

A sample of 5 ml blood was withdrawn from each child and allowed to clot at room temperature. The samples were centrifuged at 3,000 rpm and the sera were separated and stored frozen at -20 °C until used for assaying the chosen parameters using commercial kits. Serum total protein was determined by the Biuret method (Henry, 1964) and serum albumin by a modified bromocresol green binding assay (Tietz, 1995). Urine samples were also obtained and screened for PKU using the ferric chloride method (Berry *et al.*, 1958).

3. Results

Females were predominant in the studied cohort (65%). The ages of 49% of the studied children were two years or less, while 39% were between 2 and 4 years. The remaining 12% aged between 4 and 6 years. The common presentations among the studied children are presented in Table 1. It could be seen that gastrointestinal manifestations constituted most of the complaints. Deficiency manifestations like delayed growth milestones and rickets also ranked high. By reviewing family history, it was found that sibling deaths were reported in 13% of cases and mental retardation in 5%. Nine percent of the families reported consanguinity between parents. Physical examination of the children revealed congenital anomalies in 5 cases, in the form of polydactyle, cleft lip, cleft palate and ventricular septal defect. Three of these cases had consanguinity between parents.

Table 1. Age distribution of infants screened

Age	Frequency	Percent	Valid Percent	Cumulative Percent
Less than or Equal 2 years	49	49.0	49.0	49.0
Between 2 and 4 Years	39	39.0	39.0	88.0
More than 4 Years	12	12.0	12.0	100.0
Total	100	100.0	100.0	

During the course of the study, two cases of PKU were identified, based on laboratory tests, clinical presentation and family history. Both phenylketonuric infants were born in rural areas. Analysis of their family histories revealed that both infants were siblings of consanguineous parents. One of the infants had a family history of sibling deaths and the other had a family history of mental retardation.

Clinical examination of the two positive cases revealed typical presentation of PKU. Both infants suffered mild malnutrition expressed as decreased levels of serum total proteins and albumin comparable to cases of malnutrition of marasmus, kwashiorkor or other deficiencies like rickets (Table 2).

Table 2. Common clinical presentations of the studied children

Complaint	Frequency
Diarrhea	19%
Pneumonia	15%
Delayed growth milestones	13%
Rickets	12%
Vomiting	11%
Convulsions	6%
Jaundice and organomegaly	5%
Coarse facial features and skeletal deformities	2%

4. Clinical findings

The first diagnosed case was a three year old girl of a consanguineous couple. The child presented with history of delayed growth milestones, repeated vomiting and hyperirritability since birth. She was treated for hypoxic ischemic encephalopathy with seizures. There was family history of mental retardation, but no sibling deaths, convulsions or albinism. The child had frequent myoclonic jerks with drooling of saliva. There was gross microcephaly with head circumference of 37 cm. Overall motor, language and social developmental delays were noted. Brain CT scan was uneventful. Urine was tested for PKU by the ferric chloride method, and gave a positive result.

The second diagnosed case was a three and half years old boy of a sanguineous couple. Family history revealed death of 2 siblings after 1-2 weeks of birth. Clinical

examination revealed delayed developmental parameters like walking, speaking and head support (Table 3). CNS examination showed normal higher mental functions. There was generalized hypotonia with normal plantar reflex and lordosis of the lumbar spine. The urine sample which was taken was turbid and foul smelling. Screening for PKU by the ferric chloride method was positive.

Table 3. Serum levels of total proteins and albumin in the diagnosed cases of PKU as compared to ranges of cases presented with malnutrition and healthy controls in the studied cohort.

	Healthy controls	Cases with Malnutrition	Case 1 PKU	Case 2 PKU
Total protein	6.0-8.0 mg/dl	3.0-5.2 mg/dl	5.0 mg/dl	5.5 mg/dl
Serum albumin	3.5-5.5 mgldl	1.5-2.8 mg/dl	2.5 mg/dl	2.7 mg/dl

5. Discussion

The use of the ferric chloride method for detecting phenyl pyruvate in urine as a screening procedure for PKU has been proven acceptable, easy and cheap. It is suitable for screening the newborn in underprivileged areas without large medical facilities. Out of the 18,000 cases seen in the pediatric clinics of Sohag University hospital over three years, one hundred were suspected and 2 cases were confirmed to have PKU. This prevalence of 1 in 9000 is higher than what was reported by other groups. However, increases in the number of diagnosed cases over time have been reported (Bhatt *et al*., 2008; Choudhuri *et al.*, 2006; Kumta, 2005; Imamura *et al.*, 1999; Schulpis *et al.*, 1991; Wuu *et al.*, 1988). This may be attributed to advances in diagnostic technology, better coverage and reporting, and increased awareness.

Hypoproteinemia has been reported by Hanley and his co-workers (1970) in 5 of 32 infants treated with a special low phenylalanine formula. Generally, a phenylalanine-restricted diet consists of protein hydrolysate, amino acid mixtures, fruits and vegetables with minimal amount of natural animal products, usually milk, to meet the daily requirement of phenylalanine needed for early growth. In children with PKU, diet therapy could influence the immune system, not only by antigenic change, but also by producing changes in plasma lipids. It has been known to cause a marked reduction of arachidonic acid levels in both plasma total lipids and phospholipids of children with PKU during dietary intervention.

An added finding in the present study is the effect of consanguinity on the incidence of metabolic disorders, as we reported a higher incidence of parental consanguinity than other population studies. Because inborn errors of metabolism are extremely rare and random variation in their incidence is high, the diagnosis of even one extra case over a s hort time period may have an important effect on the birth prevalence of certain disorders.

In conclusion, we strongly recommend the expanded newborn screening for PKU for every baby born before discharge from the nursery for children delivered in the hospital or on first visit to the clinic for children delivered at home. Early diagnosis is important for treatment and genetic counseling. The ferric chloride method used in the present study for detection of PKU is simple, acceptable, inexpensive and can be used for screening in remote or underserved areas in the countryside.

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