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Original Articles

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Prey Selection and Feeding Rates of *Drupella cornus* (Gastropoda: Muricidae) on Corals from the Jordanian Coast of the Gulf of Aqaba, Red Sea

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Abstract

The corallivorous muricid *Drupella cornus* represents a potential threat to coral reefs. This study aimed to describe and quantify gazing activity and prey selection by *D. cornus* on corals from the Jordanian coast of the Gulf of Aqaba. Field observations revealed that *D. cornus* was mostly found on branching corals such as *Acropora* sp. and *Stylophora* sp. with an average of 13.72 ± 7.4 and 9.8 ± 9.2 individuals per colony, respectively, while only 4.0 ± 2.2 individuals per colony were found on the massive coral *Porites*. Laboratory experiments confirmed our observations in the field in terms of prey selection, where *Acropora* and *Stylophora* attracted most *D. cornus* when several coral species were placed with them in laboratory aquaria. The measured grazing rates of *D. cornus* were greatly influenced by seawater temperature, increasing by five times at 30ºC compared with 18ºC i.e., rates of 1.31 ± 0.19 and 0.27 ± 0.11 cm^2. day^{-1} $individual^{-1}$, respectively. No significant effect between *D. cornus* wet body weight and grazing rate was detected.

Keywords: *Drupella cornus*, corallivorous snails, coral, Gulf of Aqaba, reef health.

1. Introduction

Predators like the crown-of-thorns starfish (*Acanthaster planci*) and corallivorous gastropods as well as disease are common threats to coral populations in many countries around the world (Birkeland, 1989; Schumacher, 1992). Anthropogenic effects and various corallivores are recognized as important factors in structuring reef communities (Baums *et al.* 2003). During the last two decades, gastropod predation by *Drupella* spp. on corals has caused significant destruction to many reefs across the Indo-Pacific region (Turner, 1994a; Johnson and Cumming, 1995; Cumming *et al.*, 1999). Furthermore, gastropod predation, together with coral disease and bleaching, are largely blamed for declines in threatened coral species, e.g., *Acropora palmata* and *Acropora cervicornis*, in addition to interference with recruitment and prevention of juvenile growth (Miller 2001; Schuhmacher *et al*., 2002; Baums *et al*., 2003).

Three species belonging to the genus *Drupella*, i.e., *D. cornus, D. rugosa* and *D. fragum* are recognized. Of those, only *D. cornus* can be found in the Red Sea (Johnson and Cumming, 1995). *Drupella cornus* prey almost exclusively on living coral tissues (Turner, 1994b) and display outbreaks similar to those of *Acanthaster planci* (Turner, 1994a; Black and Johnson, 1994; McClanahan, 1997). Such outbreaks are thought to be due to overfishing of the natural predators and to changes in water temperature and salinity (Lam and Shin, 2006). Outbreaks of *Drupella* spp. can cause significant damage to impacted reefs. For example, it was reported that *D. rugosa* and *D. fragum* outbreaks had a significant impact on scleractinian corals with up to 35% destruction (Moyer *et al*., 1982; Boucher, 1986). *Drupella rugosa* is reported to cause severe bioerosion of certain coral species in Hong Kong (Lam and Shin, 2006). Furthermore, it was suggested that there is a correlation between the abundance of the gastropods and disease and that these can be transmitted between affected and healthy corals by the corallivores (Antonius and Riegl, 1997; Williams and Miller, 2005).

Corallivorous gastropods exhibit prey selectivity in the field, some being restricted to only a few coral prey, while others are more general (Moyer *et al*., 1982; Fujioka and Yamazato, 1983; Turner, 1994a; Shafir *et al*., 2008; Morton and Blackmore, 2009; Schoepf *et al*., 2010). Some corallivorous gastropods are less selective so that they redistribute themselves among the available prey, when the prey of choice decreases in abundance. It has also been shown that *Coralliophila abbreviata* doubled numbers on infested coral colonies with a decrease in coral cover instead of switching to another host (Baums *et al*., 2003).

The relatively small (about 27 km) coastline of Jordan is located on the north eastern part of the Gulf of Aqaba. It has a small total coral reef' area, although it has high biodiversity values that rank among the greatest ever found (Mergner *et al*., 1992; Schuhmacher *et al*., 1995). Jordanian coral reefs are described as being in a good condition (Al-Horani *et al*., 2006) with no major catastrophes recorded that have significantly affected the corals. Some localized damage has occurred through

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industrial, solid waste and ship-based discharges, coral predation and disease, dredging, sedimentation and damage associated with diving and boat anchoring (Mergner, 1981; Walker and Ormond, 1982; Schuhmacher, 1992; Mergner *et al*., 1992; Al-Moghrabi, 1996, 2001; Wilkinson, 2000; personal observations). However, the fast growing city of Aqaba is expected to increase pressure on Jordan's coral reefs and mitigation measures might become necessary in the near future.

The present study was aimed at identifying the coral genera that are influenced by *Drupella cornus* predation in a marine protected area and to study the effect of seawater temperature on the rate of predation on corals. This was done in order to evaluate grazing rates at temperatures prevailing during summer and winter periods in the Gulf of Aqaba. Coral prey selection was also studied experimentally in order to identify the species influenced by the gastropod. Furthermore, and since age categories of *D. cornus* are classified according to size (Turner, 1994b; Black and Johnson, 1994), we have studied the effect of *D. cornus* size on coral grazing rates to identify the most destructive size class. The overall objective of the study was to assess the potential of *D. cornus* to cause significant damage to coral reefs, especially during outbreaks.

2. Materials and Methods

2.1. Study site and sample collection:

Two coral reef sites located in front of the Marine Science Station were selected to collect samples of *Drupella cornus* (Fig. 1). The two sites are part of a marine protected area (classified as class-A MPA) and are characterized by high coral cover and high biodiversity (Mergner *et al*., 1992). The sites are devoid of direct human impacts. Indirect impacts relate to the neighbouring passenger port, where ship movement and solid waste disposed by passengers are partially affecting local reefs (personal observations). *Drupella cornus* individuals were collected during spring and summer using SCUBA from between 0.5-5 m depth along the coast of the Marine Science Station (ca. 700 m long). During the survey, all coral colonies present in an area were inspected for the presence of *D. cornus*. The divers used forceps to pick up individuals from infested corals. The numbers of individuals collected and the infested corals and their sizes were recorded and used to analyze the distribution and natural prey selectivity of *D. cornus* in the field. The collected *D. cornus* were immediately transferred to the laboratory and maintained in flow through aquaria irradiated with HQI lamps (12:12 light dark cycle) and fed with pieces of coral fragments.

2.2. Coral prey selection

Coral prey selection by *Drupella cornus* was studied by incubating individuals with seven coral species that are common to the Gulf of Aqaba. About 100 individuals were placed midway in a 50 L aquarium (dimensions; 60 cm X 30 cm X 30 cm) and small coral samples representing *Acropora* sp., *Stylophora* sp., *Porites* sp., *Turbinaria* sp., *Platygyra* sp., *Fungia* sp. and *Favites* sp. were placed at the edges in the same aquarium. After the movements of the *D. cornus* individuals were completed, the numbers associated with each coral sample were counted. The experiment was repeated three times and averages plotted as percentages of the total number of individuals used in each trial.

2.3. Effect of incubating seawater temperature on coral grazing

Freshly collected *Drupella cornus* individuals were used to study the effects of changing seawater temperature on coral grazing rate. Five different seawater temperatures ranging between 18°C and 30°C at three degree intervals were selected to conduct this experiment. Seawater was maintained at the desired temperature by means of a circulating thermostat. Temperatures were selected to cover the range recorded in the Gulf of Aqaba, which is between 21°C in winter and 27°C in summer (Manasrah and Badran, 2008). In addition, one point below the lowest and one above the highest recorded temperatures were tested. Preliminary tests showed that *D. cornus* is a fast grazer and, therefore, only two individuals were used to study grazing rate. Four replicates were carried out at each temperature, each one comprising two individuals and one small piece of the coral *Acropora* sp. (i.e. the preferred prey). Before the start of each experiment, both the coral and the *D. cornus* were left in seawater at the desired temperature for a few hours acclimatization. The experiments lasted until grazing (i.e. removal of the living tissue of the coral piece) was complete. The grazing rate was calculated by dividing the calculated coral surface area grazed by the time each *D. cornus* individual spent consuming it and expressed as cm^2. day^{-1}. $individual^{-1}$.

The surface area of the coral sample was measured by taking two photographs using an underwater digital camera of the two opposing surfaces. Subsequently, the surface areas of the two photographs were measured using the software Image Tool, and the sum of the two considered as the total surface area of the coral sample.

2.4. Effect of body weight on grazing rate

The collected *Drupella cornus* samples were separated according to their wet body weight into five categories; 0.1-1.0, 1.1-2.0, 2.1-3.0, 3.1-4.0 and 4.1-5.0 g. From each size category, four individuals were maintained at room temperature with a piece of *Acropora* sp. with a known surface area. The experiment lasted until complete coral tissue removal was observed and the grazing rate was calculated for each individual *D. cornus* by dividing the coral surface area by the time needed to complete the grazing. Each experiment was repeated four times and averages plotted.

Statistical analysis of the results obtained was performed using a comparison test (one way Anova at a confidence level of 95%).

3. Results

The field distribution of *Drupella cornus* with regard to the type and size of coral prey are shown in table 1. Most of the collected *D. cornus* were attached to *Acropora* sp., while *Stylophora sp*. and *Porites* sp. had fewer individuals per colony. The average number of individuals were 13.72 ± 7.4, 9.8 ± 9.2 and 4.0 ± 2.2 for *Corpora, Stylophora* and

Porites, respectively. Statistical analysis of the data revealed highly significant differences among the three coral species (P = <0.0001 between *Acropora* and *Porites*, P = 0.0094 between *Acropora*, and *Stylophora*, P = 0.0051 between *Stylophora* and *Porites*). *Drupella cornus* was found on species other than those listed in Table 1 within the study area, although *Galaxea fascicularis* and *Pocillopora damicornis* had *D. cornus* in other localities outside the study area. The depth distribution of *D. cornus* was between 0.5-5.5m for all samples collected. The shell heights of most (about 95%) collected individuals were in the range between 1.1 cm and 3.0 cm with about 75% between 1.1 and 2.0 cm and about 20% between 2.1 and 3.0 cm (Fig. 2).

Maintaining the *Drupella cornus* with seven different coral species that are common to the Jordanian coast of the Gulf of Aqaba showed that 63.5% ± 11.8 of the individuals moved towards the *Acropora* sp. The remaining individuals moved to the other coral species as follows:- 16.4% ± 7.2 towards *Stylophora* sp., 7.4% ± 3.4 towards *Porites* sp., 3.7% ± 3.2 towards *Favites* sp., 4.4% ± 1.5 towards *Platygyra* sp., 3.5% ± 0.8 towards *Turbinaria* sp., and 1.1% ± 0.9 towards *Fungia* sp. (Fig. 3). Statistical analysis of the results revealed highly significant differences between the *Acropora* sp. and all the other coral species (P <0.0001), while the only significant difference among the other species was found between *Stylophora* sp. and *Fungia* sp. (P <0.04). It was also noted that the movement of *D. cornus* was probably affected by crowdedness as some individuals, which did not find space on the preferred coral prey, were attached to the available corals in the aquarium. This is supported by the notion that tissue removal was only recorded for the *Acropora* and *Stylophora* species, while the rest of the incubated corals showed no symptoms of tissue necrosis, although there were some *D. cornus* attached to them.

The results of the effects of seawater temperature on the grazing rate of *D. cornus* on *Acropora* sp. showed that this increased steadily with increasing seawater temperature (Fig. 4). That is, the rate increased from 0.27 ± 0.11 cm^2. day^{-1}. $individual^{-1}$ at 18ºC to 1.31 ± 0.19 at 30ºC. Statistical analysis revealed significant differences among all rates except between 18ºC and 21ºC (P <0.0001).

The effect of *D. cornus* body weight on grazing rate on *Acropora* sp. is shown in Figure 5. Grazing rates for individuals in the size categories 0.1-1.0g, 1.1-2.0g, 2.1-3.0 g, 3.1-4.0 g, and 4.1-5.0 g were 0.52 ± 0.03, 0.56 ± 0.13, 0.57 ± 0.10, 0.57 ± 0.10 and 0.575 ± 0.05 $cm^2.day^{-1}.individual^{-1}$, respectively. No significant differences in grazing rate related to body weight were detected (P >0.05).

Table 1. Field distribution of *D. cornus* on the coral colonies in the study site. Only coral colonies with *D. cornus* are reported upon.

Coral type	No. of colonies sampled	Relative colony size (avg. no. of branches)	Depth range (m)	Total no. of *D. cornus*	No. of *D. cornus* per coral colony (range)	Avg. no. of *D. cornus* per coral colony
***Acropora* sp.**	160	5.22	0.5-5.5	2195	1-50	13.72 ± 7.41
***Stylophora* sp.**	28	4.46	0.5-5.5	247	1-43	9.79 ± 9.22
***Porites* sp.**	23	2.87	0.5-5.5	91	1-10	3.96 ± 2.18

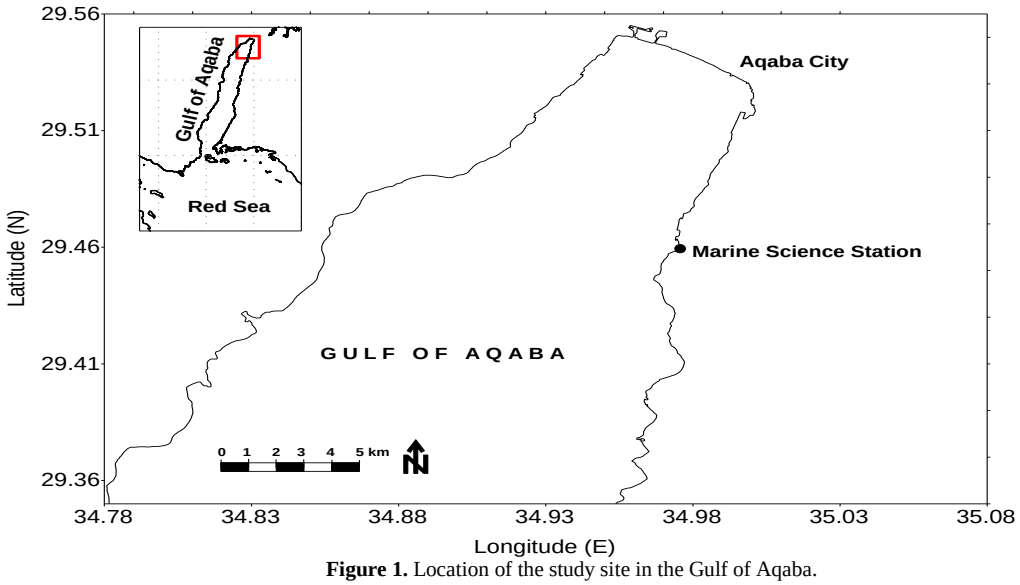

Figure 1. Location of the study site in the Gulf of Aqaba.

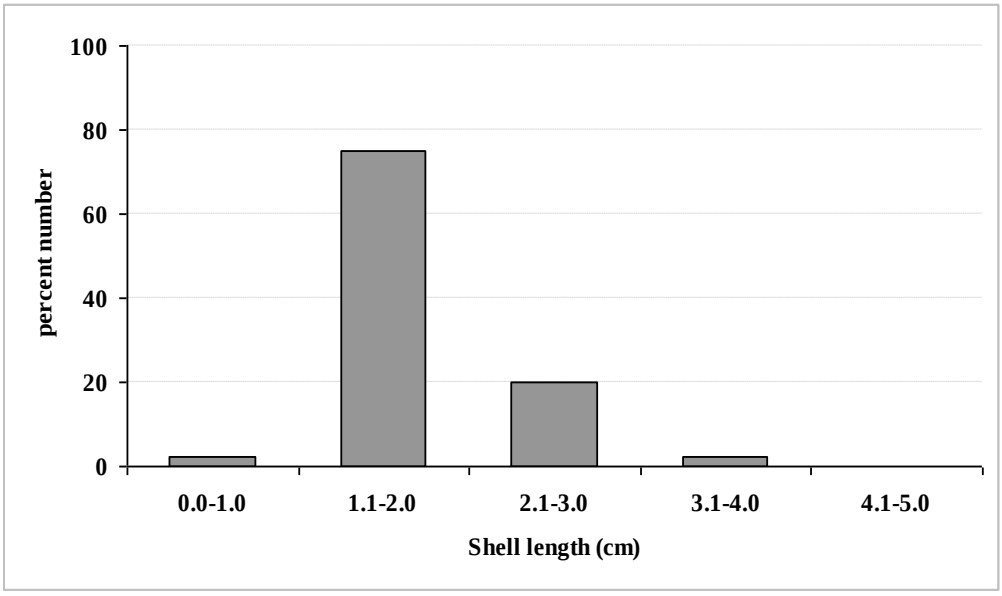

Figure 2. Shell height categories of the collected *D. cornus* individuals. The results are presented as percentages of each category for the total number of samples collected (total number of individuals collected was 2560).

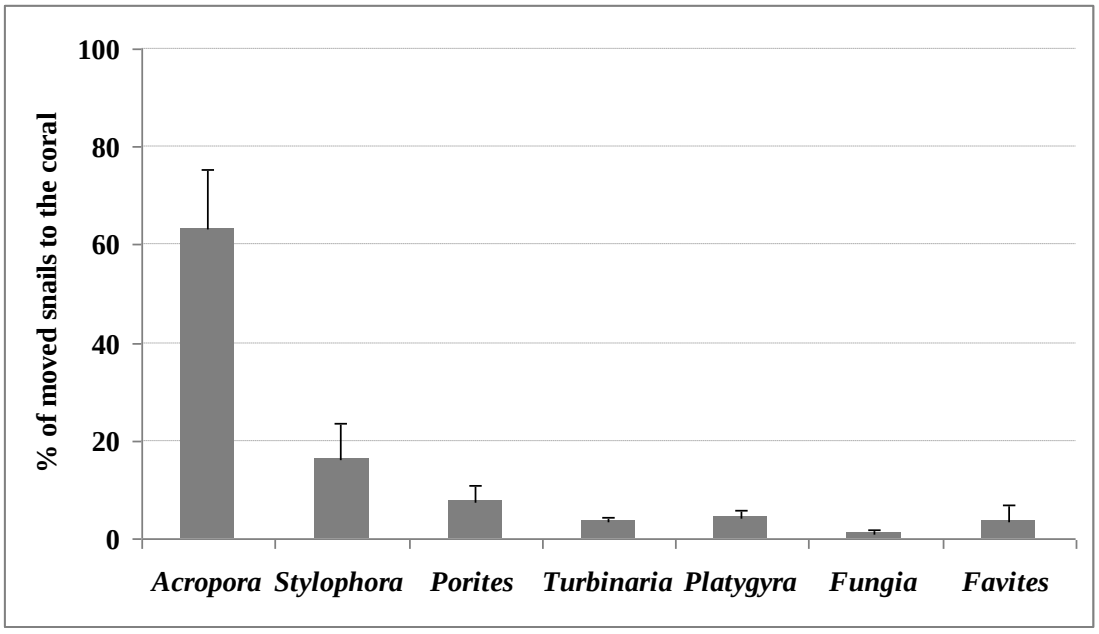

Figure 3. Percentages of the *D. cornus* individuals that moved to the different corals maintained with them. Numbers represent averages ± standard deviations.

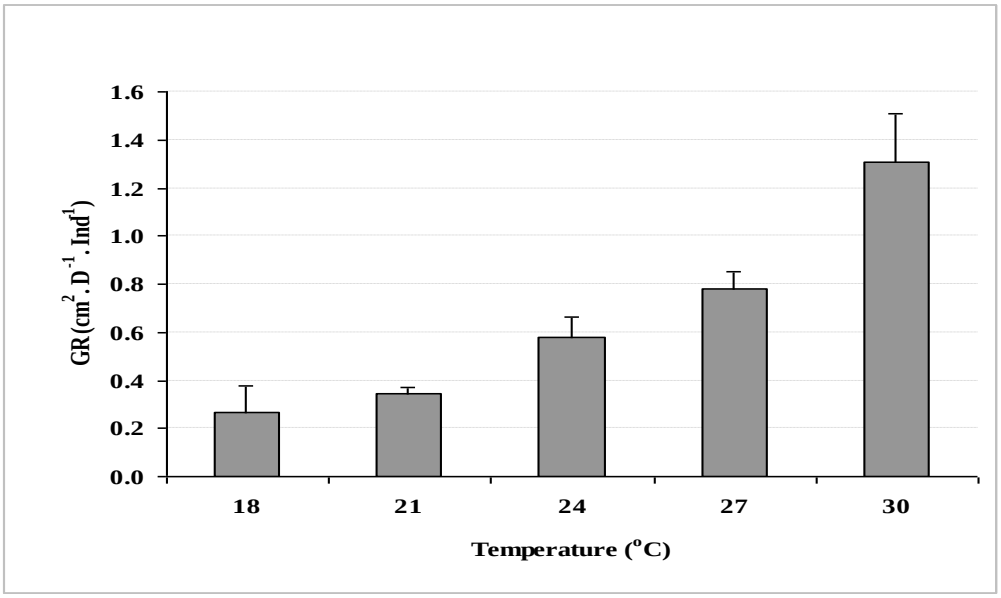

Figure 4. Grazing rates of *D. cornus* on *Acropora* sp. at different seawater temperatures. Numbers represent averages ± standard deviations.

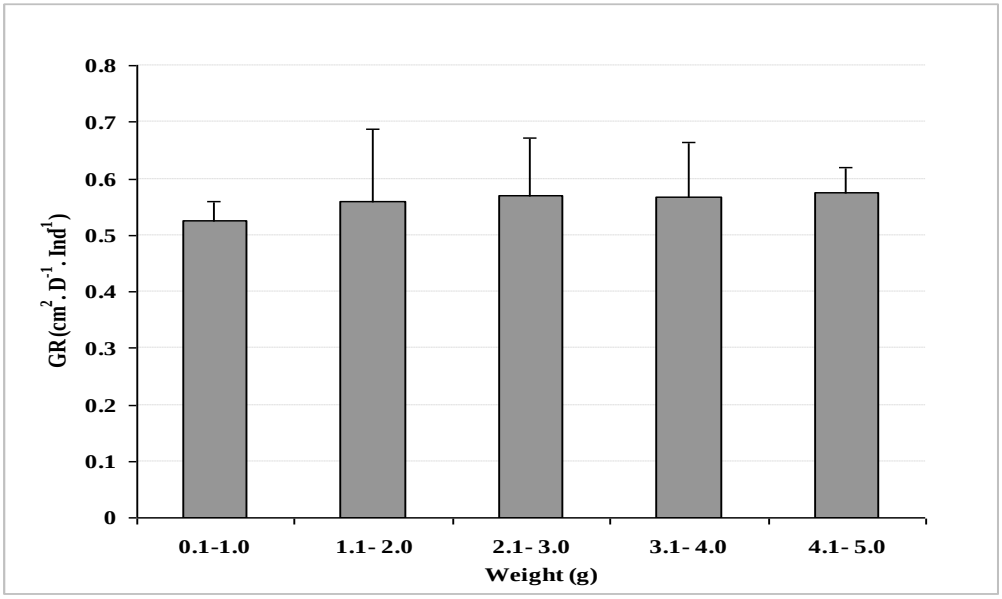

Figure 5. Grazing rates of the different body weight categories of *D. cornus* on *Acropora* sp. Numbers represent averages ± standard deviations.

4. Discussion

Analyses of the field data have indicated that *Drupella cornus* displays selectivity with respect to their preferred coral prey. Most of the collected individuals were associated with branching corals belonging to *Acropora*, suggesting that this genus is preferred over other corals. To a lesser extent, *Stylophora* was second in terms of the number of *D. cornus* individuals associated with it, while few were found with *Porites* sp., which is a massive coral (Table 1). The results obtained are in accordance with previously reported results from the Indo-Pacific and the Red Sea, where *Acropora* was always highly selected by *D. cornus* and other congeners (Turner, 1994a; McClanahan, 1997; Antonius and Riegl, 1997 and 1998; Cumming, 1999; Zuschin *et al*., 2001; Morton and Blackmore, 2009; Schoepf *et al*., 2010; Guzner *et al*., 2010). The selection of coral prey by some *Drupella* species, such *D. rugosa* was described as being complex as it changes according to the relative abundance of each coral taxon (Morton and Blackmore, 2009). Some species also display plasticity with respect to their coral prey, especially when the preferred corals are less abundant (Fujioka and Yamazato, 1983; Shafir *et al*., 2008; Schoepf *et al*., 2010), while others adjust their distributions among the preferred coral prey by increasing their numbers per coral colony (Baums *et al*., 2003).

Acropora in addition to *Stylophora* and *Porites* are important and dominant coral genera in the Red Sea (Riegle and Velimirov, 1994; Riegl and Piller, 1999). Thus, the destruction of species belonging to those genera

might lead to a significant change in the community structure of coral reefs in the area. It was suggested that the impacts of gastropod predation on reef communities will vary with coral species composition, in which case, when the preferred coral species are dominant, then the impact will be more evident on reef structure (Baums *et al*., 2003). The results of this study show that in some instances, about 50 individuals of *D. cornus* were found associated with one *Acropora* colony. This kind of aggregation has been described from Australia and elsewhere in the Red Sea (Johnson *et al*., 1993; Cumming, 1999; Zuschin *et al*., 2001). The field survey of *D. cornus* in the Gulf of Aqaba by Al-Moghrabi (1996) showed that number per square area was higher in industrial areas when compared with reserve areas. *Drupella cornus* can produce more than 150 thousand plankton veligers one month after spawning and which swim to their coral prey (Turner, 1992). Both anthropogenic impacts, such as the increased run-off, over fishing of predators such as triggerfish and increased reef damage, and natural causes such the variable larval recruitment, the existence of branching corals and a lack of competitors like *Coralliophila neritoidae*, have been suggested to explain outbreaks of *Drupella* spp. (Turner, 1994a; b; McClanahan, 1997; Baums *et al*., 2003; Guzner *et al*., 2010).

Age categories of *Drupella* have been classified according to shell height, recruits a being < 1.0 cm, juveniles < 2.0 cm, and adults >2.0 cm, which is about 2.5-3.5 years old (Turner, 1994b; Black and Johnson, 1994). In the present study, about 75% of the collected individuals had a shell height of between 1.1 cm and 2.0 cm and about 20% had a shell height of between 2.1 cm and 3.0 cm. According to the above classification of the age categories, the majority of the *D. cornus* collected for this study were, thus, juveniles with about one fifth being adults with an age of ~2 years. The recruits (<1.0 cm) and larger individuals (>3.0 cm) occurred in small numbers suggesting that the former had not been produced in large numbers during the preceding year, i.e., 2007, and that the latter might have been subjected to predation by, for example, trigger fish.

The maintenance of *D. cornus* in the aquarium together with different species of corals showed that *Acropora* sp. was preferred followed by *Stylophora* sp., both being branching corals. This observation agrees with the field observation, where the *Acropora* sp. had the highest number of *D. cornus* followed by *Stylophora* sp. In a similar experiment, it was found that *D. rugosa* preferred *Acropora* and *Montipora* over other corals like Leptastrea, *Pavona*, *Platygyra* and *Favia* (Morton *et al*., 2002). The results obtained suggest that some corals may have attraction factors for *D. cornus*. Kita *et al*. (2005) isolated montiporic acids from extracts of *Montipora* sp., and which were shown to be potent feeding attractants for *D. cornus*. Similarly, Ochi *et al*. (1992) have isolated calicogorgins from the gorgonian *Calicogorgia* sp. and which posses lethal and repellent qualities against *D. fragum*. It is, therefore, suggested that *Acropora* sp. and *Stylophora* sp. may contain attractants for *D. cornus*, while the rest of the studied corals may not, although biochemical analyses are needed to confirm this. Prey preferences in the field are suggested to depend on factors such as coral genus, colony shape, susceptibility to predators, influence of host tissue nutritional quality and/or secondary metabolite contents, and genetic differences (Baums *et al*., 2003; Schoepf *et al*., 2010). Schoepf *et al*. (2010) suggested that *Acropora* corals provide the best combinations of food and shelter for *D. cornus* and thereby determine its distribution pattern in the field. This hypothesis does not explain why similar coral species like *Pocillopora* or *Seratophora* are much less preferred by *D. cornus* although both of them are highly branching and should provide the necessary food and shelter. The prey selection exhibited by *D. cornus* might lead to changes in reef community structure, especially when outbreaks happen and might also lead to significant decreases in the number of the preferred coral prey as suggested for *Acropora palmata* (Miller 2001; Baums *et al*., 2003). The normal feeding activities of *D. cornus* might not cause significant damage to corals, but when outbreaks occur ecosystem changes might result, especially when this is added to other stressors such as bleaching, diseases and pollution.

When *D. cornus* was maintained with the coral *Acropora* at different seawater temperatures relatively high grazing rates were recorded, especially at warmer temperatures. Although, the mean surface seawater temperature in the Gulf of Aqaba is usually about 21ºC during winter and about 27ºC during summer (Manasrah *et al*., 2004), extreme temperatures might decrease to 18ºC in winter and increase to 30ºC in summer. The results obtained here showed that the grazing rate of *D. cornus* increased by about 5 fold at 30ºC compared with that at 18ºC (Fig. 4). This indicates that the feeding activity of *D. cornus* significantly increases in summer as compared with winter. This observation has important implications for reef health, especially when outbreaks happen as it can lead to destruction of the preferred prey on a coral reef (Turner, 1994a). It is possible that the grazing rate increases at warm temperatures because the corals become stressed as suggested for *D. rugosa*, which is attracted to mechanically stressed corals (Morton *et al*. 2002). The field observations made by Moyer *et al*. (1982) have shown that *D. fragum* caused destruction to *Acropora* reefs during summer with about one third of the reef being impacted in southern Japan (Moyer *et al*., 1982). Similarly, it has been reported that the number as well as the feeding activity of *D. rugosa* showed significant seasonal differences being significantly greater in summer (Morton and Blackmore, 2009). Such results have implications for reef conservation, which must consider mitigation measures during summer when outbreaks of *D. cornus* occur as it was reported that the direct removal of the snail helps preserve the infested corals (Miller 2001).

Body weight is herein shown to have no significant effect on the grazing rate of *D. cornus* (Fig. 5). The reason for this is unknown, but it is reported that the growth rate of *D. cornus* is higher for juveniles than it is for adults (Black and Johnson, 1994). This might be because of the higher energy demands of juveniles.

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Effects of Rosemary (*Rosmarinus officinalis*) on Lipid Profile of Diabetic Rats

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Abstract

The purpose of this study was to determine the hypoglycemic activity of the aqueous extract perfusion of rosemary (*Rosmarinus officinalis* Linn.) in normal and streptozotocin-induced diabetic rats. The sugar level and lipid profile were investigated in plasma of normal and streptozotocin-induced diabetic rats treated with rosemary for four weeks. Diabetic rats exhibited an increase in the levels of sugar, total cholesterol, triglycerides and low density lipoprotein cholesterol (LDL-cholesterol), and a decrease in the level of high density lipoprotein cholesterol (HDL-cholesterol). The administration of rosemary shows a decrease by 20% in sugar level, 22% in total cholesterol, 24% triglycerides, and by 27% in LDL-cholesterol, and an increase by 18% in HDL-cholesterol. The findings of this study indicate that the administration of rosemary resulted in a better lipid profile and decreased blood sugar level in both normal and diabetic rats.

Keywords: Rosemary, *Rosmarinus officinalis*, streptozotocin, rat, diabetes, lipid profile, antioxidant; antidiabetic.

1. Introduction

Diabetes mellitus (DM) is a serious health problem being the third greatest cause of death all over the world, and if not treated, it is responsible for many complications affecting various organs in the body. Diabetes mellitus results in hyperglycemia and is characterized as type 1 in absolute insulin deficiency or type 2 in insulin resistance due to receptor insensitivity to endogenous insulin (ELHilaly *et al.*, 2007).

Proper nutrition is essential for anyone living with the diabetes in particular in type 2 diabetes mellitus. Control of blood glucose levels is only one goal of a healthy food plan for people with diabetes. A diet for those with diabetes should also help achieve and maintain a normal body weight as well as prevent heart and vascular disease, which are frequent complications of diabetes (Melzig and Funki, 2007). Diabetes remedy that is gaining popularity today is herbal treatment, with a variety of plant-derived preparations being promoted as capable of controlling blood sugar levels, in fact, herbal treatment for diabetes is not new. Plants and plant extracts were used to combat the disease as early as 1550 B. C., with as many as 400 before the development earlier this 21st century of effective medications to control diabetes (Tapsell *et al*., 2006).

Hyperlipidemia is a complication associated with diabetes mellitus (Miller *et al*., 2002) due to qualitative and quantitative abnormalities in lipoproteins. Chronic hyperglycemia in diabetes leads to over production of free radicals and these contribute to the development of diabetic nephropathy (Sharma *et al*., 2006). Atherosclerosis and coronary heart disease are the major health problems (Braunwald, 1997; Breslow, 1997; Law, 1999). A number of epidemiological investigations have shown a clear association between dietary saturated fat and atherosclerosis (Shekelle *et al*., 1981; Posner *et al.*, 1991).

Moreover, many studies have shown that elevated total or low density lipoprotein (LDL) cholesterol in the blood are powerful risk factors for coronary heart disease (Law, 1999), whereas high HDL-cholesterol: LDL-cholesterol ratio may protect against coronary heart disease (Sheten *et al.*, 1991; Castelli *et al.*,1992).

The use of herbs as medicines has played an important role in nearly every culture, including Asia, Africa, Europe and the Americas (Wargovich *et al.*, 2001). Herbal medicine is based on the premise that plants contain natural substances that can promote health and alleviate illness. Several herbs can help to reduce blood sugar, high blood cholesterol, provide some protection against cancer and stimulate the immune system. Furthermore, a diet in which culinary herbs are used generously to flavor food provides a variety of active photochemical that promotes health and protective against chronic diseases. Additionally, several commonly used herbs have been identified by the National Cancer Institute in USA as possessing cancer-preventive properties. These herbs include members of the *Allium* sp. (garlic, onions and chives), members of the *Labiatae* (mint) family (basil, mints, oregano, rosemary, sage and thyme), members of

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Zingiberacae family (turmeric and ginger), (Steinmetz and Potter, 1991).

Rosemary (*Rosmarinus officinalis* Linn.) and mint (*Labiatae*) family are common household plant grown in many parts of the world. They are commonly used as a spice and flavoring agent in food processing (Saito *et al*., 2004). Also, rosemary is used as an antispasmodic in renal colic and dysmenorrheal, in relieving respiratory disorders and to stimulate hair growth. Extract of rosemary relaxes smooth muscles of trachea and intestine, and has choleretic, hepatoprotective and antitumergenic activity. Moreover, rosemary constituents have a therapeutic potential in the treatment or prevention of bronchial asthma, spamogenic disorders, diabetes mellitus, peptic ulcer, inflammatory diseases, hepatotoxicity, atherosclerosis, ischemic heart diseases, cataract, cancer and poor sperm motility (Al-Sereiti *et al.*, 1999; Masuda *et al.*, 2002; Sotelo-Fleix *et al.*, 2002; Osakabe *et al.*, 2004).

The present study was designed to evaluate the effects of rosemary, on blood lipid profile and blood sugar of rats. Blood lipid profile includes, triglycerides, high density lipoprotein cholesterol (HDL), and low density lipoprotein (LDL).

2. Materials and Methods

2.1 Animals

Forty male albino rats weighing between 140 and 155 gm were procured from Department of Medical Technology, Zarqa Private University, Jordan. The animals were housed in a well ventilated 12 hrs light and 12 hrs dark cycles. The animals were divided into 2 equal groups: group I, normal rats as control, group II streptozotocin-induced diabetic rats treated animals (150 mg/kg intraperitoneally).

2.2 Rosemary extraction:

Fifty gm of rosemary were soaked in 150 ml hot water (88 ºC) in water bath for 3 hrs, filtered with capron silic cloth and the filtrate (which was 45 ml) was stored in dark bottles in refrigerator at (4 ºC). These procedures were repeated when needed. Each group of rats was orally administrated with 0.5 ml (1.11gm/ml) of rosemary extract daily.

2.3 Blood sampling

Blood was collected from group1 and group 2 three days after streptozotocin treatment (zero time) and also 4 weeks later after rosemary administrated. The blood was collected from eyes of all groups in heparinized tubes. Plasma was separated and kept in freezer till the time of assay.

2.4 Biochemical analysis

The following analyses were carried out: Glucose, total-cholesterol, Triglycerides, LDL-cholesterol and HDL-cholesterol using kits from Syrbio, France.

2.5 Statistical analysis

Statistical analyses were done utilizing the computer data processing (SPSS, version 14). A probability value (*P*) of <0.05 was considered to be statistically significant.

3. Results

Table 1 depicts the level of plasma glucose, total-cholesterol, triglycerides, HDL-cholesterol and LDL-cholesterol in both control and experimental rats at zero time, Rosemary has no significant influence on plasma glucose level and lipid profile of normal rats. Table 2 shows mean value of glucose and lipid profile after rosemary treatment for 4 weeks. There was a 20% reduction in glucose level, 22% in total-cholesterol, 24% in triglycerides, 27% in LDL-cholesterol, whereas HDL-cholesterol increased 18%. The differences were significant at (*P*<0.01) and (*P*<0.05), respectively.

Table 1. Depicts the level of plasma glucose and lipid profile at zero time without rosemary.

	Normal Rats Mean± SD (mg/dl)	**Diabetic Rats Mean± SD (mg/dl)**
Glucose	85. ±6.1	296.2±5.2
Total cholesterol	92.3±3.3	189.6±4.1
triglycerides	88±4.42	201.7±9.2
LDL-cholesterol	60.2±5.5	87.7±8.5
HDL-cholesterol	51.3±2.2	42.4±3.4

Table 2. Depicts the level of plasma glucose and lipid profile at the end of 4 weeks with rosemary.

	Normal Rats with Rosemary Mean± SD (mg/dl)	Diabetic Rats with Rosemary Mean± SD (mg/dl)
Glucose	78. 9±6.3	236.8±9.6*
Total- cholesterol	80.8±4.5	147.4±2.9*
triglycerides	71.7±6.3	152.7±9.1**
LDL-cholesterol	52.2±4.2	63.6±2.8**
HDL-cholesterol	62.15±8.2	49.5±4.3*

** highly Significant at(P <0.01)

* significant at (P <0.05)

4. Discussion

Oral administration of rosemary leaf extract caused significant declines in the blood levels of triglycerides, total cholesterol, LDL-cholesterol, but increased HDL-cholesterol. Moreover, it seemed that rosemary leaf extract had a hypolipidemic potential. This may be an indication of progressive metabolic control of rosemary leaf extract on mechanisms involved in elimination of the lipids from the body, this hypolipidemic properties have been confirmed in many plant species and plant products in medicinal use (Kono *et al.*, 1992; Naidu and Thippeswamy, 2002; Devi and Sharma, 2004). The most important constituents of rosemary are caffeic acid and its derivatives such as rosmarinic acid. These compounds have antioxidant effect (Al-Sereiti *et al.*, 1999). A variety of phenolic compounds, in addition to flavonoids, are found in fruit, vegetables and many herbs. The phenolic compounds (such as caffeic, ellagic, and ferulic acids, sesamol, and vanillin) inhibit atherosclerosis (Decker, 1995). In addition to a well documented role in reverse cholesterol transport, HDL-cholesterol has recently been recognized to have several other important cardio protective properties including the ability to protect LDL from oxidative modification (Nofer *et al*., 2002).

Yasser et *al.*, 2010 agree with our study, found that the rosemary extract has been targeting the hormone sensitive lipase (HSL) would be the common link between the two

metabolic effects. In fact, HSL has been extensively studied for its effects on the metabolic switch between glucose and free fatty acids (FFAs) as an energy source.

Fuhrman *et al.* (2000) reported that polyphenols glabridin (derived from licorice), rosmarinic acid or carnosic acid (derived from rosemary), as well as garlic (which contains a mixture on natural antioxidants) inhibited LDL oxidation in a dose-dependent manner. Moreover, several studies showed that plant extracts lowered LDL oxidation (Ramirez-Tortosa *et al.*, 1999; Doi *et al.*, 2000; Naidu and Thippeswamy, 2002). However, the present data demonstrated that consumption of rosemary can lead to reduction in the risk of hyperlipidemic symptoms and heart diseases. It can be concluded from presented results that rosemary was associated improvement in the lipid profile. Further studies are needed to purify the bioactive constituents of the extract and use the purified constituents for bioassay-directed experiments either in hyperlipidemic or non-hyperlipidemic organisms.

Suzuki *et al*. (2002) suggested that intake of vegetables and fruits rich in carotenoids might be protective factor against hyperglycemia. Flavonoids are functional constituents of many fruits and vegetables. Some flavonoids have antidiabetic properties because they improve altered glucose and oxidative metabolisms of diabetic states.

Platel and Srinivasan (1997) reported that vegetables are among numerous plant adjuncts tried for the treatment of diabetes mellitus, green leafy have shown the beneficial hypoglycemic influence in both experimental animals and humans.

On the other hand our data show that in all rosemary treated groups mean HDL-cholesterol level was increased as compared to the control group. In this respect, Tapsell *et al*. (2006) reported that the antioxidant properties of rosemary are of particular interest in view of the impact of oxidative modification of low-density lipoprotein cholesterol in the development of atherosclerosis. Herbs and spices have an important role in dietary flavonoids intake. Chamomile, onions, rosemary, sage and thyme have high flavonoids contents, but there is little evidence apart from epidemiological studies to support a direct cardiovascular health benefit from these herbs and spices.

Olmedilla *et al*. (2001) reported that basal diets containing goadaid reduced the levels of LDL-cholesterol, TC and TG. She observed also that HDL-cholesterol level was raised in diet with goadaid. Exposure to high fruit and vegetable content in diet increases antioxidant concentrations in blood and tissues and potentially protects against oxidative damage of cells and tissues.

Conclusions are that Rosemary is one of the dietary components that is known as safe and used every day in our food products. Our animal study confirms that rosemary extracts exert a hypoglycemic effect and improve the lipid profile. These effects should be studied further in human volunteers and diabetic patients.

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Bioactive Compounds from a Polypore Fungus *Ganoderma applanatum* (Per s. ex Wallr.) Pat.

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Abstract

Bioactive chemical compounds G1 and G2 were isolated, purified and identified from fruit bodies of the wood-rot polypore fungus *Ganoderma applanatum* collected from *Tamarix aphylla* trees in southern Iraq. The identification of these two compounds by using GC-Mass and H^1NMR was confirmed. Solubility of both compounds in different solvents was tested and the toxicity of these two compounds against human blood showed a negative result. The molecular weights of the purified G1 and G2 compounds are 336 kd and 360 kd, respectively. The chemical formula of G1 is $C_{20}H_{34}O_4$ which belongs to Tanin group while G2 is $C_{21}H_{28}O_2$ and belongs to terpenoides group. The antimicrobial bioactivities of the purified compounds against bacterial strains *E. coli* and *S. aureus* and against selected dermatophytic fungal isolates were tested using a disc diffusion agar method. The minimal inhibitory concentration (MIC) was also applied. Purified G1 and G2 compounds exhibited good bioactivities against the tested bacteria but did not show any activity against the selected dermatophytes.

Keywords: Bioactive compounds, dermatophytes, *Ganoderma applanatum*, polypore fungus.

1. Introduction

The research interest to explore new antimicrobial agents from fungi is continued. Nonetheless, previous investigations reported that several fungal mushrooms exhibit bioactive chemical components against pathogenic bacteria and fungi (Jonathan and Fasidi, 2003; Anke *et al.*, 2004; Ketting *et al.*, 2005; Al-Fatimi *et al.*, 2006; Jonathan and Awotona, 2010) . *Ganoderma* is a genus of polypore fungi (Basidiomycetes) often grows on tree parks causing white rot (Phillips 1983). Among the *Ganoderma* species, *G. pafeifferi* showed to have an antimicrobial compound known as Ganonycin (Mothana *et al.*, 2000). It has been reported that *Ganoderma* species was used as antitumor and antioxidant, and in some other medicinal therapy (Ulrike *et al.*, 2005). *G. applanatum*, however, is a distinctive species relative to the members of the genus and it grows on parks of *Tamarix aphylla* trees in Southern Iraq. According to our knowledge so far a little information is available about the production of bioactive secondary metabolites by this fungus. This report elucidates interesting chemical compounds extracted, purified and identified from fruit bodies of *G. applanatum* as a bioactive agents tested against a selected isolates of bacteria and dermatophytic fungi.

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2. Materials and Methods

2.1. Fungal Mushroom culture

Fruit bodies of the polypore *Ganoderma applanatum* were collected from the trunks of *Tamarix aphylla* in southern Iraq during spring 2008. In the laboratory, small pieces (0.5 cm long) were cut from the fruit body, surface sterilized with 10 % Sodium hypochlorate for 3 min, washed with sterile distilled water and placed on Malt Extract Agar (MEA) in Petri dishes. Plates were incubated at 25 °C for two weeks. After cultivation, the mycelium was removed from the agar medium surface and amended into a liquid culture medium consisted of (40 g glucose, 10 g malt extract, 4 g yeast extract in 1L distilled water). Then the mycelium culture was transferred into a fermentation medium in 1L volume conical flasks as described by Anke *et al.* (2004) and incubated at 25 °C on a rotary shaker for 3 weeks.

2.2. Extraction, isolation and purification

The fungal culture was filtered on Whatman No. 1 filter paper, the pH was adjusted at 3 using 2N HCl. The filtrate was extracted three times with ethyl acetate (1:1 vol) using a separating funnel. The organic layer was collected and dehydrated with Na_2SO_4 then placed in Petri dishes and dried at room temperature. Thin layer chromatography (TLC) was applied for the isolation of the extracted metabolites using Silica gel of 2x 10 cm (Silica gel GF243, Merck, Germany) and Rf values were measured. Purification of the extracted compounds was made on silica gel column chromatography (Silica gel G-60, Merck, Germany). A further purification of fraction compounds was made by a column chromatography

method **(**Fig.1**)**. The purity of compounds was verified according to the described method (Zure, 2001). The identification of the purified compounds was made by using GC-mass and HNMR techniques.

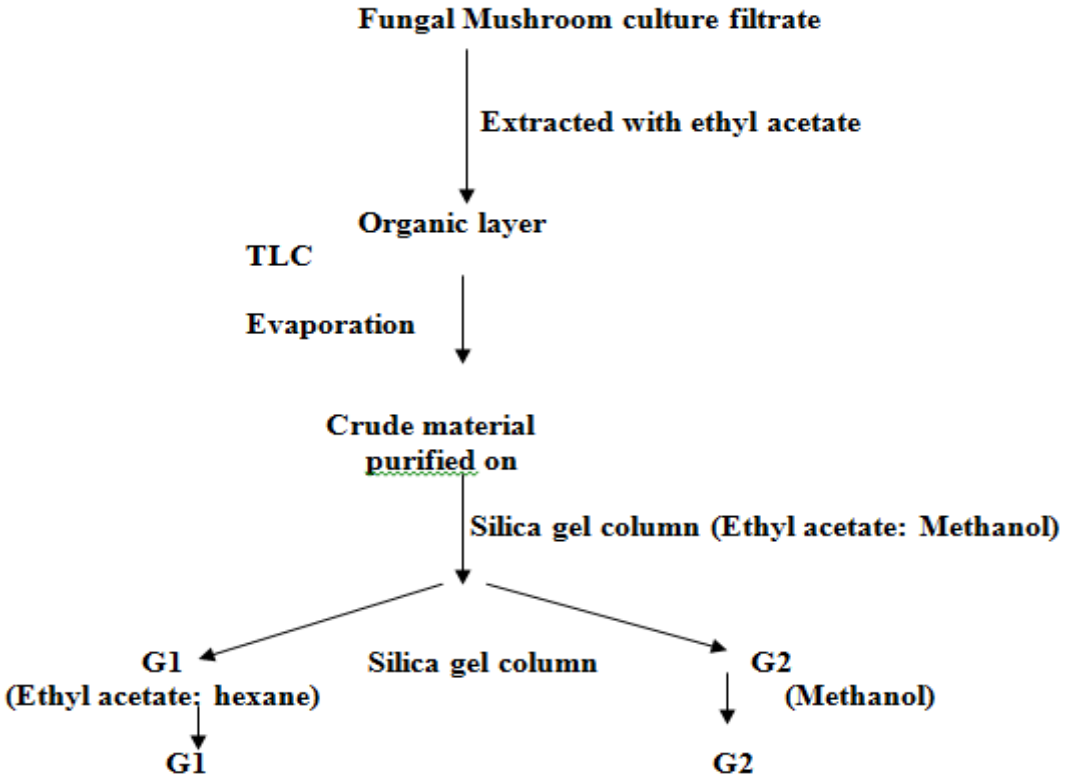


Figure 1. Steps of extraction and purification of bioactive compounds G1 and G2 from *G. applanatum*.

2.3. Bioactivity Test

Discs diffusion agar method (Casals, 1979) was used to examine the antimicrobial activity of the purified compounds. Two strains of bacteria; *Escherichia coli* (ATCC 25922) and *Staphylococcus aureus* (ATCC 25923) were used for this purpose. 2.5 mg of the dried fungal extract was dissolved in 1 ml of dimethyl sulfuoxide (DMSO) solvent used as stock solution for this test. Discs of 0.6 mm diameter Whatman No1 filter paper were sterilized and soaked in the fungal extract and placed on plates containing Muller-Hinton Agar (MHA) medium inoculated with 0.1 ml suspension of bacterial strains by streaking method. Similarly, the antifungal bioactivity of these compounds was also tested against three isolates of dermatophytic fungi (*Microsporum canis*, *M. gypseum* and *Trichophyton mentagrophytes*) using Sabouraud dextrose agar (SDA) medium. Bacterial cultures were incubated at 37°C and dermatophytic cultures were incubated at 27°C. Fungal cultures were obtained from the Basrah General Hospital, Dermatology section.

2.4. The minimum inhibitory concentration (MIC) test

The MIC values were determined by the standard serial dilution assay (McGinnis, 1980) using serial dilutions of the fungal extract (100, 50, 25, 12, 6.5, 3.13, 1.56, 0.78, 0.39, 0.2, 0.1, 0.05, 0.025 µg/L). The MIC values in this assay were indicated by the absence of bacterial or fungal growth at the minimal concentration of the compound. Emmons Sabourauds dextrose broth (ESDB) medium was used for this test.

2.5. Cytotoxic test

Cytotoxicity of the purified compounds was examined by using human RBC following a previously described method (Xian-guo and Ursula, 1994).

2.6. Solubility test

The solubility of the bioactive compounds in various solvents (ethyl acetate, ethanol, methanol, chloroform, hexane, DMSO and water) was carried out.

2.7. 2.7 Identification of bioactive compounds

Ultra violet (UV) spectrum (LKB-Sweeden UV), Infra-red spectrum (IR) (Pye-Unicam sp 3-3005 UK), Gas chromatography Mass (GC) and H^1NMR methods were applied for the identification and determination of the molecular weights, chemical formula and structure of the purified bioactive compounds. .

3. Results

Fungal extract showed two spots on TLC referred as G1 and G2 with Rf values of 0.70 and 0.50, respectively. Solubility test of G1 and G2 indicated that both components are insoluble in the water but they are soluble or partially soluble in most of the other examined solvents **(**Table 1).

Table 1. Solubility test of purified compounds G1 and G2 in various solvents

Purified component	Ethyl acetate	Methanol	Ethanol	Chloroform	Hexane	Water	DMSO
G1	Soluble	Partially soluble	Soluble	Soluble	Partially soluble	Non-soluble	Soluble
G2	Partially soluble	Soluble	Soluble	Soluble	Partially soluble	Non-soluble	Soluble

Ultra violet (UV) spectra showed that the absorbency value of G1 compound was about three fold higher than G2 compound (Fig. 2).

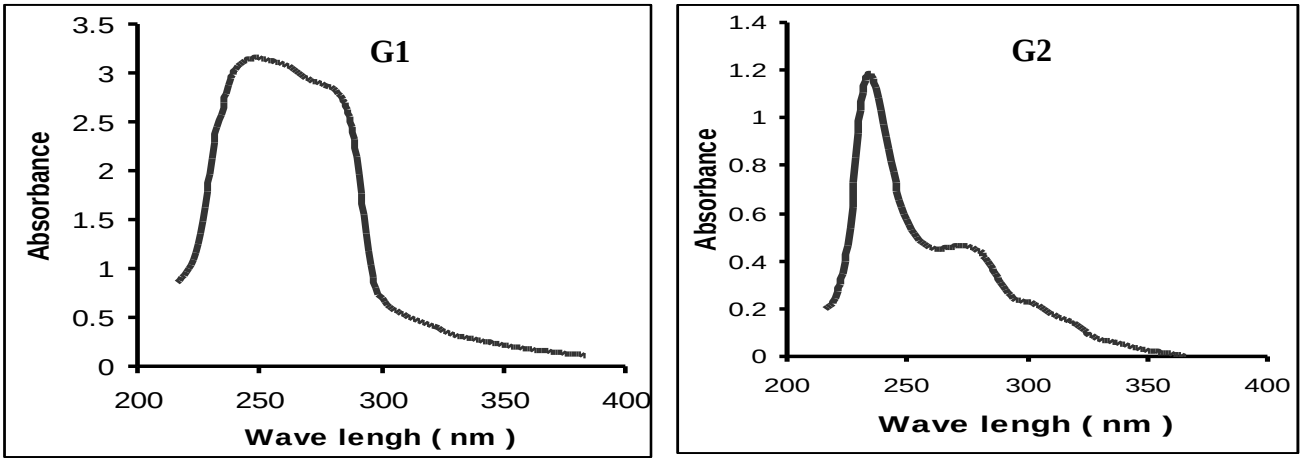


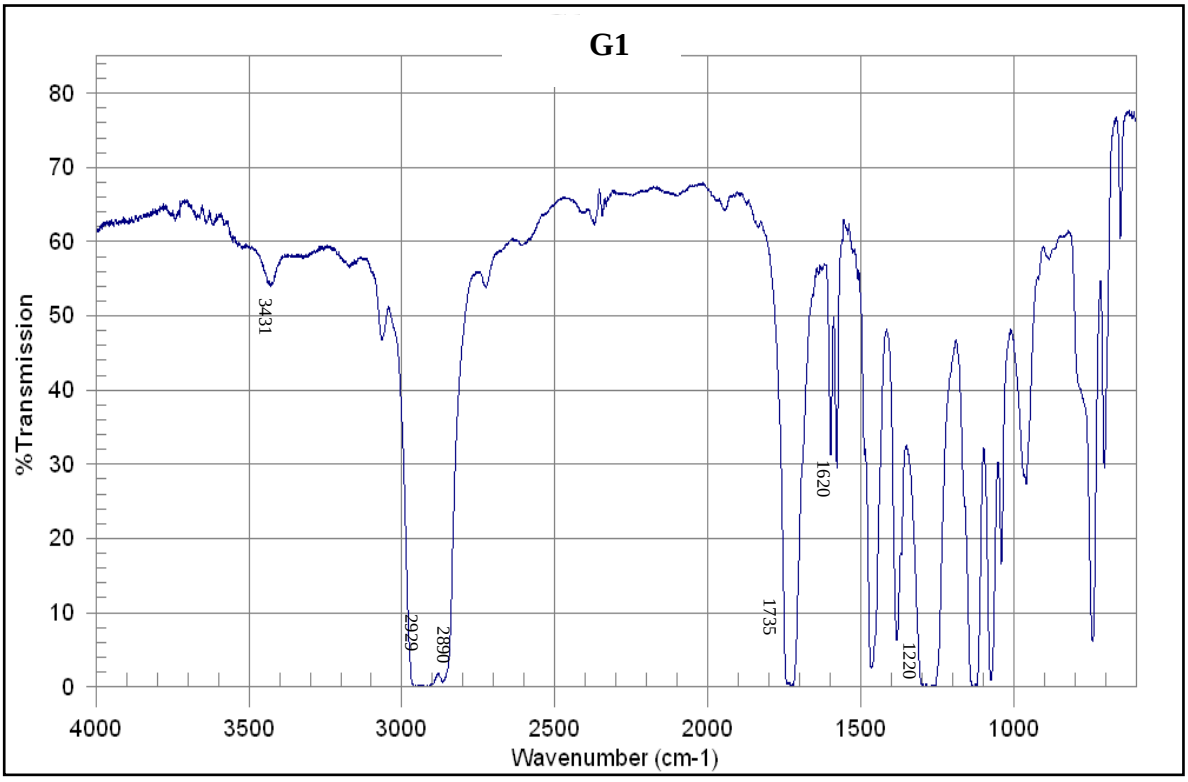
Figure 2. Absorbency of purified compounds (G1 and G2) from *G. applanatum.*

Both G1 and G2 components did not show any toxicity by using human RBC test. IR spectrum revealed that G1 and G2 composed of various functional molecules structure (Table 2).

Table 2. Infra Red spectra showed the absorbency bands of different chemical functional groups composed the purified G1 and G2 components from *G. applanatum*

Functional groups	G1	G2
N-H , O-H	3431	3400
	Weak band	Strong band
CH,CH2 ,CH	2890-2929	2900-2950-
	Strong band	Weak band
C=O	1735	1650
	Strong band	Strong band
C=C	1620	1400
	Very strong band	Weak band
C-O	1220	1260
	Very strong band	Weak band

Each of the purified compound exhibited different spectra bands representing different chemical functional groups (Fig. 3).



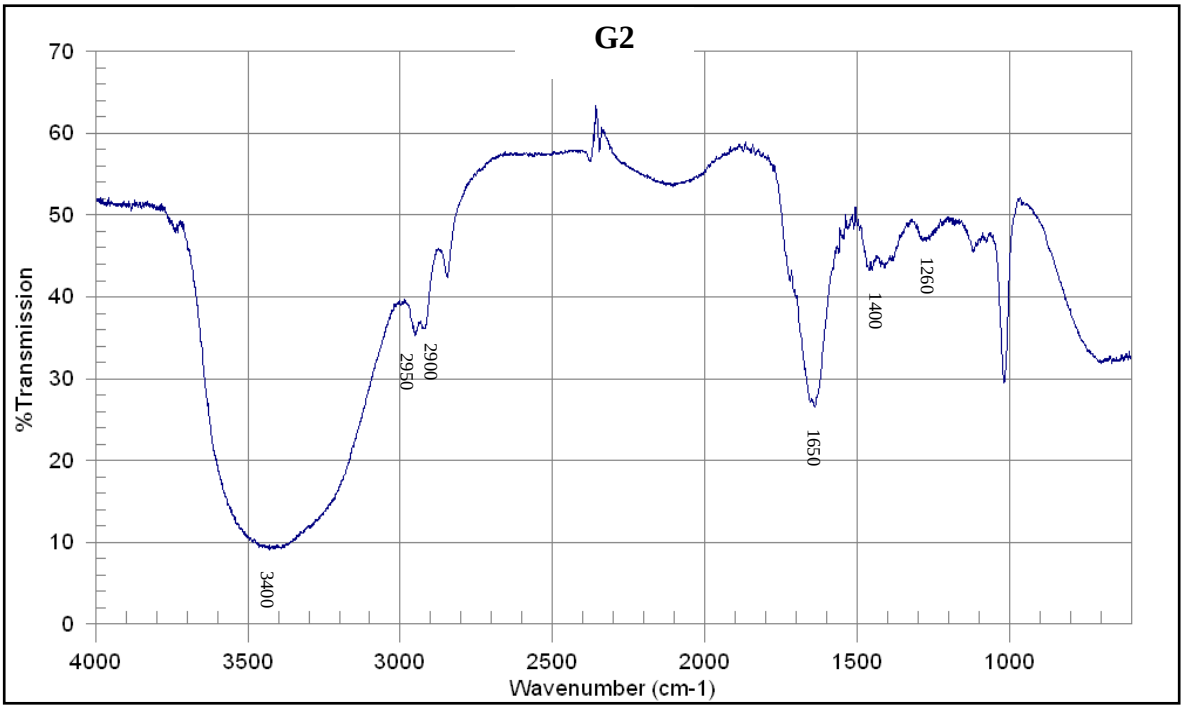


Figure 3. Infra Red spectra of the purified G1 and G2 compounds from *G. applanatum*

Based on GC- Mass and H^1 NMR methods, apparently that the molecular formula of G1 compound is **$C_{20}H_{34}O_4$** (Fig. 4) and its chemical structure is: **[**(19,19a-dihydroxy-2-methyl-2,3,4,5,6,7,8,9,10,11,12,13,14,15 tetradecahydrobenzo[b] [1] oxacycloheptadecin-17(19aH) with a molecular weight 334 Kd. This indicated that G1 compound is more related to Tanin group by comparing its spectroscopic data with available literature (Al-Fatimi *et al.*, 2006; Mothana *et al.*, 2000) (Fig. 5).

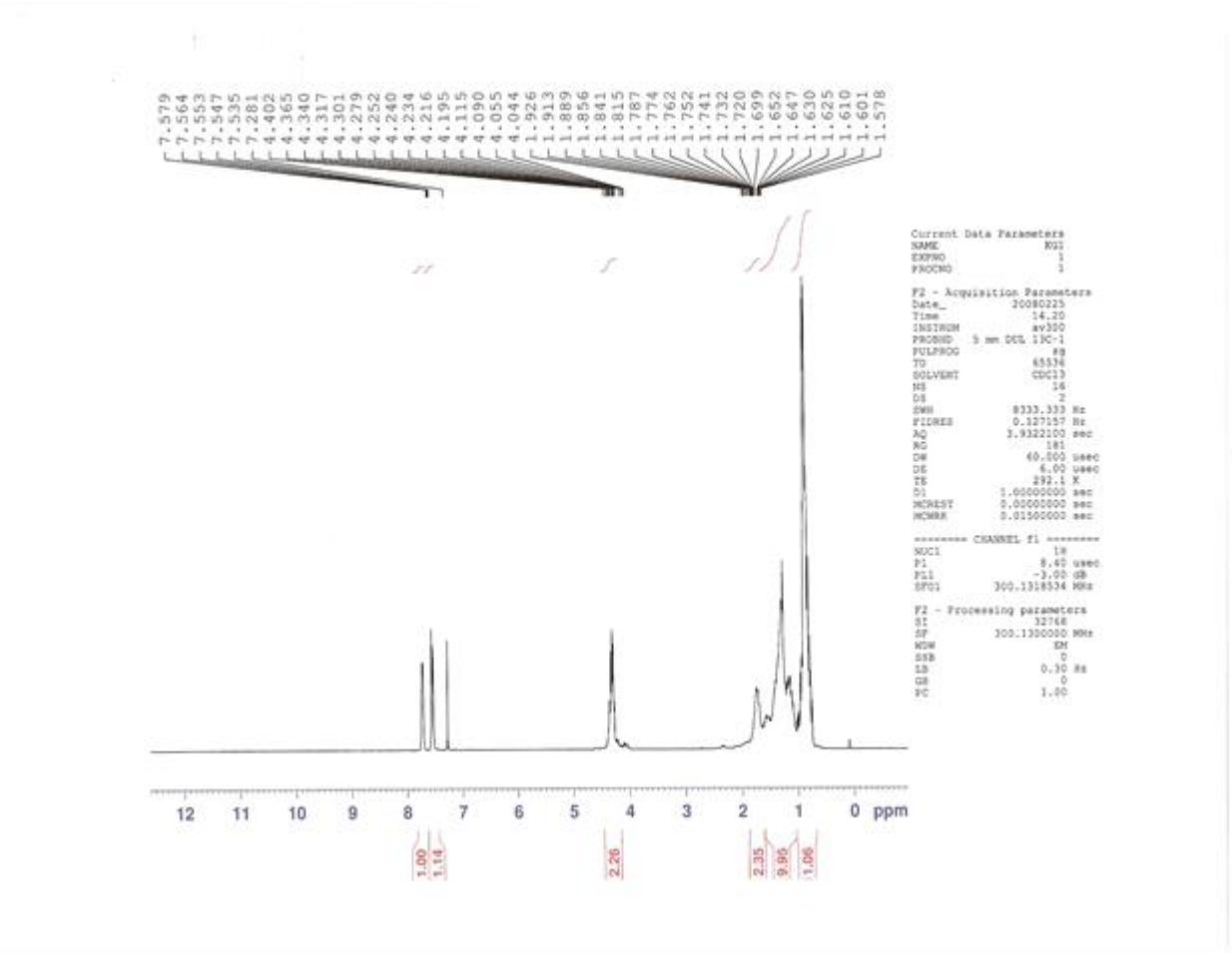


Figure 4. H^1NMR spectra of the purified compound G1 from *G.applanatum*

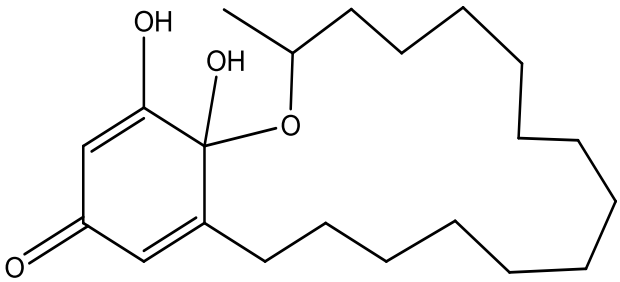


Figure 5. Chemical structure of G1 compound isolated from *G. applanatum.*

While G2 chemical formula is $\mathbf{C_{21}H_{28}O_2}$ (Fig. 6) with a molecular weight 360 kd. Its chemical structure is: **[**(2-(2-(2,5-dihydroxyphenyl) ethylidene)- 11-hydroxy-6,10-dimethylundeca-5,9-dienoic acid and its belongs to Terpenoides group (Fig. 7).

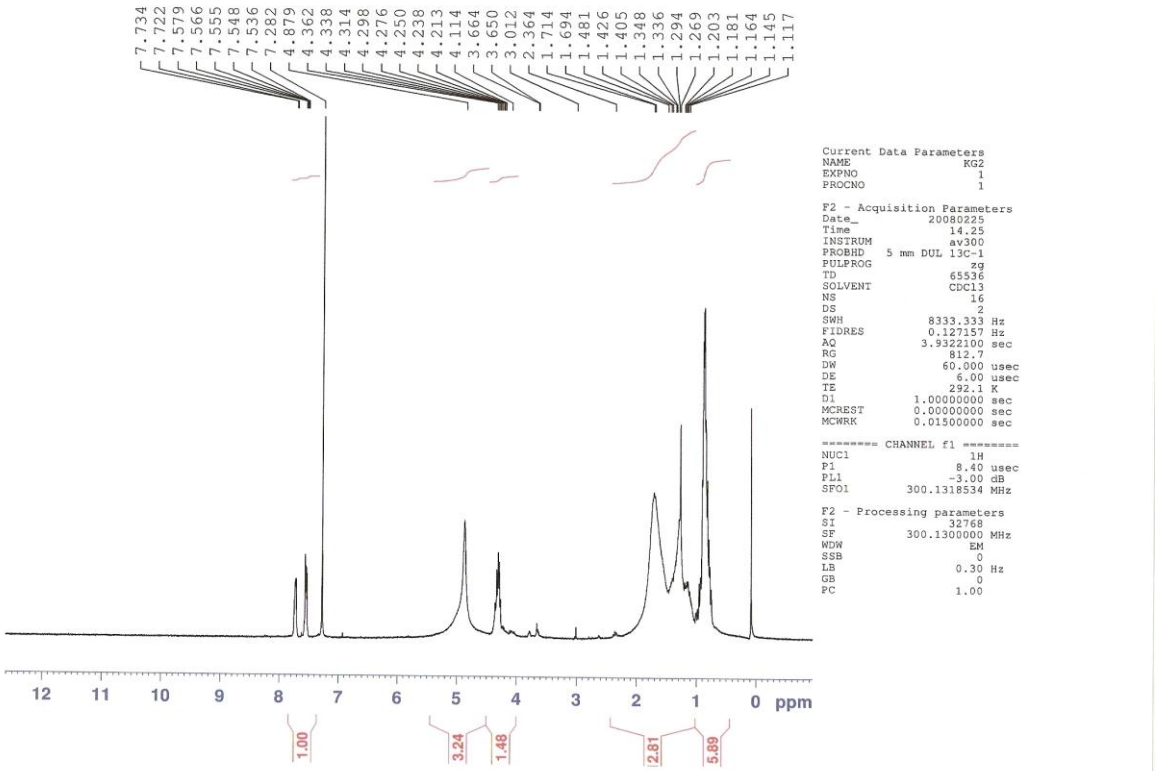


Figure 6. H[1]NMR Spectra of the purified compound G2 from *G. applanatum*

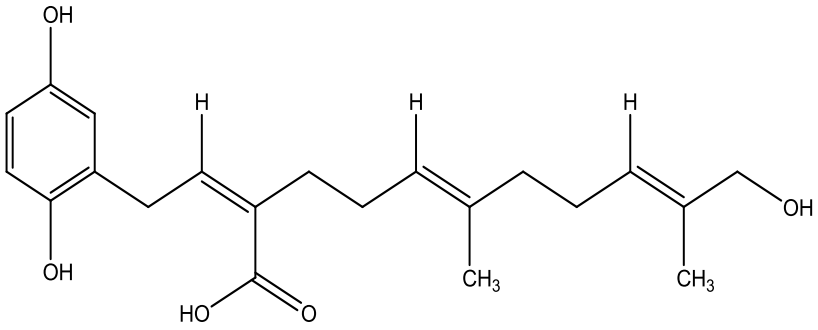


Figure 7. Chemical structure of G2 compound isolated from *G. applanatum*

Both G1 and G2 components did not show any toxicity by using human RBC test. The MIC value of the bioactive compounds G1 and G2 was 6.25 µg/L for both *E. coli* and *S. aureus*. While the two compounds were not active against the selected dermatophytic fungal isolates as they did not show a growth inhibition at any of the concentrations used. A clear zone inhibition of 23 mm diameter was observed for the crude extract against both bacterial strains E. *coli* and *S. aureus.* The inhibition zones diameters, however, exhibited by the purified G1 and G2 compounds were higher reaching to 26 mm and 29 mm for *E. coli* and *S. aureus*, respectively (Fig. 8).

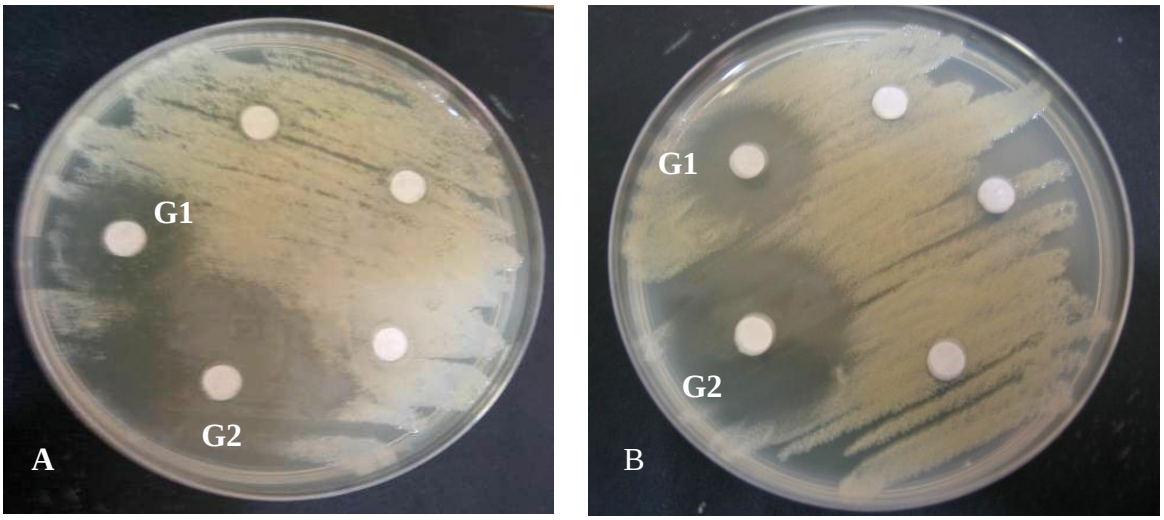


Figure 8. Inhibition zones by the purified G1 and G2 compounds against. *E. coli* (A) and *S. aureus* (B).

4. Discussion

Fungi in general and mushrooms in particular are a good source for antimicrobial products (Janes *et al.,* 2007). A few reports on the bioactive secondary metabolites of *G. applanatum* are available. This basidiomycetes species is not a rare polypore fungus living on trunks of some trees over the world (Phillips, 1983) and in southern Iraq, however, no phytochemical investigation so far has been carried out on mushrooms in this region. Our present study revealed that purified extract of *G. applanatum* exhibited an inhibitory action against both *E. coli* and *S. aureus.* These findings are in concomitant with the previous study (Roberts, 2004) who reported that *G. applanatum* produces steroides compounds which are active against G-negative and G-positive bacteria. The purified G1 compound is more likely to be chemically related to tannin group while G2 compound is more related to terpenoides group based upon their chemical structural verification by using HNMR and GC-mass spectra and agreed with the previous studies (Al-Fatimi *et al.,* 2006; Mothana *et al.,* 2000). It has been stated that ascomycetes and basidiomycetes fungi often produce terpenoides compounds (Anke, 1989). The bioactivity of G1 and G2 against the test bacteria can be attributed to the presence of tannin and terpenoide compounds. Also various chemical compounds have been screened in some species of *Ganoderma* (Bojana *et al.,* 2000). Apparently, the purified G1 and G2 compounds are not effective against the tested dermatophytic fungal isolates since no growth inhibition was observed. These results support the earlier findings (Roberts, 2004; Samania *et al.,* 2001). Although, Smala *et al*. (2003) stated that *G. annulare* produces applanoxidic acid compound which showed a weak activity against the dermatophyte *T. mentagrophyes.* The antibacterial activity of the purified compounds in this study was significantly increased compared with the activity of crude extract. A similar results were reported for some other mushrooms extract (Jonathan and Fasidi, 2003; Jonathan *et al.,* 2008; Olawuyi *et al.,* 2010). Seemingly, the isolated G1 and G2 compounds from *G. applanatum* are a good bioactive agents and promising to be used as an antibacterial. Nevertheless, there is still more fungi need to be examined for their potentiality against bacteria and pathogenic fungi.

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Susceptibility of Males and Females of Cucumber Fruit Fly, *Dacus ciliatus*, to Various Insecticides in the Laboratory Conditions

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Abstract

The cucumber fly, *Dacus ciliatus* Loew., is a destructive pest of cucurbit crops in some regions of Iran. Susceptibility of adults of *D. ciliatus* to various insecticides (deltamethrin, dimethoate, trichlorofon, spinosad, acetamiprid, and malathion) was investigated. The insecticide toxicity was tested through contact method in three stages (males only, females only, and a mixture of males and females). The results showed that the used insecticides excellently affected on males and females of *D. ciliatus* through contact toxicity. Also, their effect on the mixture of males and females was acceptable. In addition, dimethoate and deltamethrin were better than the others. The LC_{50} values of these two were less than 1 mg L^{-1}. Also, except for acetamiprid, LC_{50} vales of all other insecticides on males and females were close.

Keywords: *Dacus ciliatus*, LC_{50}, chemical control, deltamethrin, dimethoate, trichlorofon, spinosad, acetamiprid, malathion

1. Introduction

Fruit flies (Diptera: Tephritidae) are among the most important pests worldwide. The genus *Dacus* causes severe damage to fruits and vegetables in Asia. The cucumber fly, *Dacus ciliatus* Loew., is a major pest of cucurbits in some countries (Azab *et al.*, 1970; Nagappan *et al.*, 1971). *D. ciliatus* is a pest of the most of eastern, southern, and central Africa, Arabian Peninsula, Pakistan and India. Its color is orange, with facial spots. The costal band in it is apically extended to form an apical spot and a basal sloping spot. There are two black spots in abdomen particularly in females (White and Elson-Harris, 1994). In Iran, this pest is a major pest of cucumber, watermelon and cantaloupe. Also milkweed and colocynth are other hosts of this pest in Iran (Arghand, 1983).

The used insecticides in this study were deltamethrin, dimethoate, trichlorofon, spinosad, acetamiprid and malathion. A pyrethroid compound, deltamethrin (1R,3S) [α-cyano(3-phenoxyphenyl) methyl]- 3- (2, 2-dibromo-ethenyl)- 2, 2-dimethylcyclopropanecarboxylate) kills insects through contact and ingestion. Its mode of action has not been known though; pyrethroids affect neuroactivity by delaying the closing the channels of sodium (Corbett *et al.*, 1984).

Acetamiprid ((E)-N1-[(6-chloro-3-pyridyl) methyl]-N2-cyano-N1-methylacetamidine) is neonicotinoid that has high activity and is used to controlling insects of various orders on a broad range of plants, particularly vegetables and fruits (Roberts and Hutson 1999; Tomlin 2000). Spinosad (mixture of 50–95% (2*R*,3a*S*,5a*R*, 5b*S*,9*S*,13*S*,14*R*,16a*S*,16b*R*)-2-(6-deoxy-2,3,4-tri-*O*-methyl-α-L-mannopyranosyloxy)-13-(4-dim-ethylamino-2,3,4,6-tetradeoxy-β-D-erythropyrano-syloxy)-9-ethyl-2, 3,3a,5a,5b,6,7,9,10,11,12,13, 14,15,16a, 16b-hexadecah-ydro-14-methyl-1*H-as*-indaceno[3,2-*d*]ox acyclododecine-7,15-dione and 50–5% (2*S*,3a*R*,5a*S*, 5b*S*, 9*S*, 13*S*, 14*R*, 16a*S*,16b*S*)-2-(6-deoxy-2,3,4-tri-*O*-methyl-α-L-manno-pyranosyloxy)-13-(4-dimethylamino-2,3,4,6-tetradeoxy-β-D-erythropyranosyloxy)-9-ethyl-2,3,3a,5a, 5b,6,7,9,10, 11, 12,13,14,15,16a,16b-hexadecahydro-4,14-dimethyl-1*H-as*-indaceno[3,2-*d*]oxacyclododecine-7,15-dione) is a selective insecticide based on a fermentation product of the soil bacterium actinomycete (*Saccharopolyspora spinosa*) (Miles and Dutton,2000). Malathion, as organophosphorus insecticide, is most used insecticide in agriculture. Its mode of action is anticholinesterase (Sharma *et al.*, 2005). Low toxicity of malathion on mammalian and its low price have changed it as a good choice for control of fruit flies (Rossler 1989; Steiner *et al.*, 1961). Trichlorfon is another organophosphorus insecticide. It is widely used in agriculture as a selective insecticide based on its mode of action (inhibitor of acetylcholinesterase) (Staples *et al.*, 1976).

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The fruit fly *D. ciliatus* is important in Iran and it is a major pest in many provinces of Iran. The main method for controlling this pest and other fruit flies is chemical control in Iran. Therefore, applying the effective insecticide for this method can be efficient and useful. In this study, we investigated the contact effect of various insecticides (deltamethrin, dimethoate, trichlorofon, spinosad, acetamiprid, and malathion) on *D. ciliatus* in three stages (effect on males, on females, and on a combination of males and females). LC_{50} values of these insecticides were calculated separately.

2. Materials and Methods

2.1. Insect stock

Dacus ciliatus colony was collected from cucumber fields of Varamin in the east of Tehran, Iran in early October of 2008. For this, the infested small cucumbers, *Cucumis sativus*, having larvae of this pest, were collected and transferred to laboratory. The cucumbers were placed in the trays (1×1m) with their bottoms covered by loam soil, 1cm in thickness, until leaving the larvae. For preventing the escape of the adults, the trays were covered by plastic cages, too. A sugar solution (10%) was used for feeding the adults in the cages. Insect rearing and all bioassay tests were performed at 25 ± 2 ℃ and 65 ± 5% relative humidity under a 12:12 (L:D) photoperiod in the growth chamber.

2.2. Insecticides

The formulated insecticides, used in the present study, were spinosad [Tracer 240 SC (AI)] deltamethrin [Decis 2.5% EC (AI)], dimethoate [40% EC (AI)], trichlorofon [Diptrix 80% SP (AI)], acetamiprid [Mospilan 20% SP (AI)], and malathion [50% EC (AI)].

2.3. Bioassay tests

The bioassay tests of this study were performed in three sections (Bioassay on males only, on females only, and on a mixture of males and females). For bioassay tests, the contact toxicity was used. All concentrations of insecticides were prepared in water. As a control, water was used. For bioassays, Petri dishes (9 cm in diameter) were sprayed with various concentrations of insecticides, and, then, they were permitted to dry at room temperature for 2 h. Ten 5 day-old adult insects were placed in each Petri dish after catching by aspirator. In the test on mixture sexes, 5 individuals were selected from each sex. A sugar solution (10%) was used to feed the insects. These tests were repeated 4 times. Mortality was recorded 24 h after treatment. The data were analyzed by Proc Probit using SAS software (SAS Institute 1997). This method for all tests was alike.

3. Results

3.1. Toxicity on males

Table 1 shows the values of LC_{50} of various insecticides on males of the cucumber fly. Values of LC_{50} of tested deltamethrin, dimethoate and trichlorfon were 0.39, 0.24 and 1.57 mg L^{-1}, respectively. LC_{50} values of spinosad, acetamiprid and malathion were 8.03, 6.87 and 2.28 mg L^{-1}, too. Results of bioassay showed that the dimethoate LC_{50} was the lowest compared to others. On the other hand, the susceptibility to spinosad was lower than others. Toxicity ratio of dimethoate on males was 1.62, 6.54, 33.45, 28.65 and 9.5 folds higher than deltamethrin, trichlorfon, spinosad, acetamiprid and malathion, respectively.

3.2. Toxicity on females

In table 2, the effects of the mentioned insecticides on females of cucumber fly are shown. Values of LC_{50} of deltamethrin, dimethoate, trichlorfon, spinosad, acetamiprid and malathion on *D. ciliatus* females were 0.52, 0.4, 2.4, 8.52, 19.88 and 3.57 mg L^{-1}. The toxicity of dimethoate on females was the highest compared to others. It was 0.76, 6, 21.3, 49.7 and 8.92 folds higher than deltamethrin, trichlorfon, spinosad, acetamiprid and malathion. It is clear that toxicity of acetamiprid is the lowest on females. Also, comparing the toxicity of these insecticides on males and females is shown in fig. 1. Figure 1 shows that males of *D. ciliatus* were more susceptible than females. This difference was clearer in acetamiprid.

3.3. Effect on mixture of males and females

Table 3 displays the effect of insecticides on mixture of 50% males: 50% females of *D. ciliatus*. Similar to the results of table 2, the highest susceptibility of adults was showed in dimethoate compared to others (table 3). LC_{50} values of deltamethrin, dimethoate, trichlorfon, spinosad, acetamiprid and malathion on adults were 0.45, 0.28, 1.95, 8.20, 11.50 and 2.85 mg L^{-1}, respectively; their lowest toxicity was seen in acetamiprid.

4. Discussion

In this study, the effectiveness of various insecticides belonging to organophosphorus, pyrethroids, biorationals and neonicotinoides (deltamethrin, dimethoate, trichlorofon, spinosad, acetamiprid and malathion) on male, female and both sexes of *D. ciliatus* was investigated. Aetamiprid was selected because of its safe effect on human beings and trichlorofon due to its selectivity on Diptera. This is the first research on the toxicity of these insecticides to investigate *D. ciliatus*; hence, the results were compared with the results on other insects.

Our results showed that dimethoate had the lowest toxicity on males, females, and both males and females. On the other hand, LC_{50} values of spinosad and acetamiprid were higher than the others. Also a pyrethroid insecticide, deltamethrin, has a good toxicity on this pest compared to malathion. Similar to this, Malakov *et al.* (2001) reported that toxicity of a pyrethroid insecticide (fenpropathrin) was higher than malathion. Using broad spectrum insecticides causes adverse effects on natural enemies, natural resource and human health. Also, secondary pest outbreaks in this condition (Emden and Peakall 1996). Hence, replacing these compounds with low side effect insecticides such as spinosad might be important. Vargas *et al.* (2003) stated that spinosad is a good replacement for DDVP and naled for killing the male oriental fruit flies and melon flies (Dipter: Tephritidae).

Table 1. Susceptibility of *Dacus ciliatus* males exposed to various insecticides in the laboratory conditions

	n[a]	df	LC_{50} (mg L^{-1})	Slope ± SE	χ^2	Toxicity Ratio
Deltamethrin	300	6	0.39 (0.29-0.55)	1.58 ± 0.23	3.32	1.62
Dimethoate	300	4	0.24 (0.13-0.28)	1.82 ± 0.28	1.09	1
Trichlorfon	300	5	1.57 (1.32-1.85)	1.89 ± 0.15	4.2	6.54
Spinosad	300	4	8.03 (2.94-11.64)	3.26 ± 1.26	2.04	33.45
Acetamiprid	300	4	6.87 (3.31-11.07)	1.5 ± 0.33	0.65	28.65
Malathion	300	4	2.28 (0.90-3.27)	3.79 ± 1.3	2.2	9.5

n[a]: number of subjects

Table 2. Susceptibility of females of *Dacus ciliatus* exposed to various insecticides in the laboratory conditions

	n[a]	df	LC_{50} (mg L^{-1})	Slope ± SE	χ^2	Toxicity Ratio
Deltamethrin	300	6	0.52 (0.38-0.75)	1.48 ± 0.22	2.8	0.76
Dimethoate	300	4	0.4 (0.25-0.53)	1.89 ± 0.41	1.1	1
Trichlorfon	300	5	2.4 (2.02-2.80)	2 ± 0.17	2.4	6
Spinosad	300	4	8.52 (1.46-12.26)	3.69 ± 1.58	1.9	21.3
Acetamiprid	300	4	19.88 (7.30-12.10)	2.8 ± 0.75	0.74	49.7
Malathion	300	4	3.57 (1.87-6.24)	2. 9 ± 1.18	2.61	8.92

n[a]: number of subjects

Table 3. Susceptibility of mixture of males and females of *Dacus ciliatus* exposed to various insecticides in the laboratory conditions

	n[a]	df	LC_{50} (mg L^{-1})	Slope ± SE	χ^2	Toxicity Ratio
Deltamethrin	300	6	0.45 (0.36-0.57)	1.51 ± 0.16	3.7	1.6
Dimethoate	300	4	0.28 (0.21-0.35)	1.71 ± 0.21	2.04	1
Trichlorfon	300	5	1.95 (1.72-2.18)	1.92 ± 0.11	5.44	6.94
Spinosad	300	4	8.20 (5.04-10.60)	3.45 ± 0.98	3.71	29.28
Acetamiprid	300	4	11.50 (7.34-15.76)	1.76 ± 0.33	2.3	41.07
Malathion	300	4	2.85 (1.99-3.76)	3.18 ± 0.8	2.65	10.17

n[a]: number of subjects

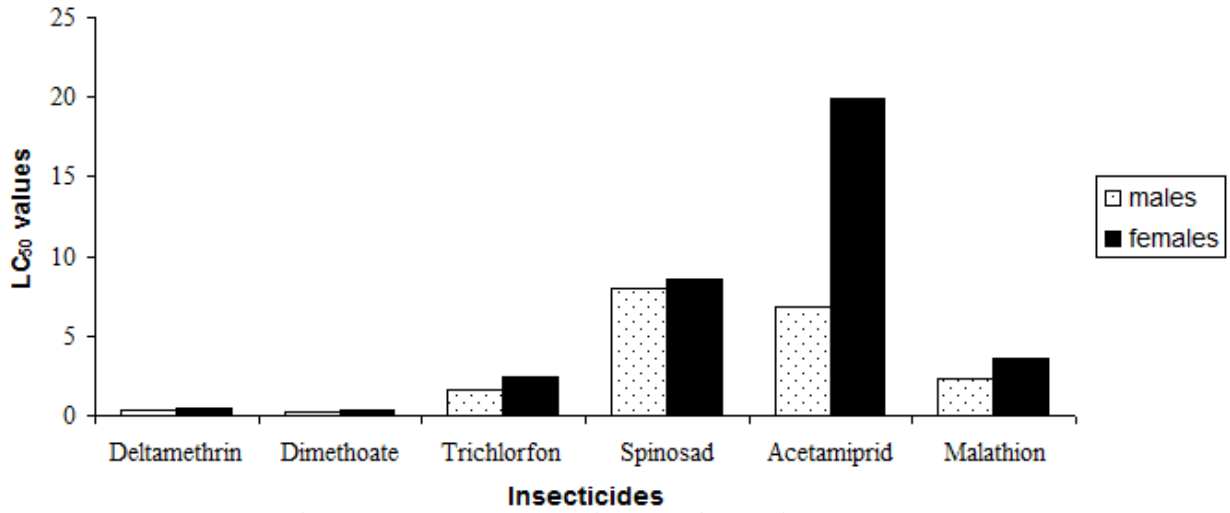

Figure 1. Comparing LC_{50} values of various insecticides on mixture of males and females of *Dacus ciliatus* in the laboratory conditions.

King and Hennessey (1996) underlined the effect of spinosad on males and females of Caribbean fruit fly, *Anastrepha suspense* (Loew) (Diptera:Tephritidae) and reported that spinosad is a good candidate for controlling it. Barry *et al.* (2004) investigated the effect of neonicotinoids (imidacloprid and acetamiprid) on a fruit fly, *Rhagoletis mendex*. They reported that acetamiprid, unlike imidacloprid, had no knock down property on that pest. Similar to this research, Olszak and Maciesiak (2004) found that acetamiprid had a good effect on blueberry maggot fly, *Rhagoletis cerasi* (Diptera: Tephritidae). Similar to the current results, Hu *et al.* (2000) stated that dimethoate is a good choice for controlling the females of *Rhagoletis pomonella* (Diptera: Tephritidae). Khan and Khattak (2000) stated that trichlorofon and malathion decreased populations of melon fruit fly, *Bactrocera cucurbitae* (Coq) and muskmelon fruit fly, *Cucumis melo* (L.) compared to the untreated group.

In Conclusion, the insecticides used in this study excellently affected males and females of *D. ciliates*; these insecticides can be useful for their chemical control. Among these insecticides, deltamethrin and dimethoate were better than others. The LC_{50} values of these two were <1 and therefore, can be important for selecting the best choice. Also, except acetamiprid, the efficacy of insecticides on males and females was approximately similar.

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Histological Changes in Tissues of Trachea and Lung Alveoli of Albino Rats Exposed to the Smoke of Two Types of Narghile Tobacco Products

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Abstract

Undoubtedly, narghile smoking has become a common social practice in the Arab region, and a growing phenomenon in the whole world. This study is an attempt to reveal the effects of narghile smoking on the cellular level, through exposing a group of experimental albino rats to the smoke of two types of narghile tobacco-derived products: flavored (moassal) and unflavored (tumbak), for three months on a daily basis, using a specially designed smoking machine. The most prominent histological changes were an abnormal proliferation in the epithelium of trachea, disruption of its cilia, and a marked hyperplasia in the connective tissue of lung alveoli. Finally, further research should be done to give definitive conclusions about the product with an overall stronger effect. However, based on our experiment, smokers could be advised not to smoke on a daily basis and in poorly ventilated areas.

Keywords: Narghile smoking, trachea, lung alveoli, moassal, tumbak, cilia.

1. Introduction

Water-pipe (narghile) is a generic name which refers to any apparatus involves the passage of smoke through water before inhalation (Al-Safi *et al*., 2009). Water-pipe smoking is currently considered a fashionable way of tobacco leaves consumption, especially among the present-day water-pipe smokers including trendy youth, university students, and even high-school-aged children, although it used to be as a pleasurable pastime of older and retired people (Onder *et al*., 2002; Knishkowy and Amitai, 2005; Neergaard *et al*., 2007).

Primarily, there are two types of narghile tobacco products: the flavored one which could be either moassal (also known as tobamel) or jurak, and the unflavored type, called tumbak (or ajamy). Both types are tobacco-based; the flavored type contains lesser amount of tobacco than tumbak. Tumbak is the one that is purely made of moistened shredded tobacco leaves, usually soaked in water before being squeezed and packed in the bowl of the narghile (Chaouachi, 2009). In addition to tobacco, moassal contains molasses, honey, or other syrups, together with glycerol, and flavoring essences (Chaouachi, 2009; Chaouachi, 2010).

Upon passing over the charcoal and through the tobacco, the heated air becomes loaded with the combustion products of charcoal, as well as a variety of products from the heated tobacco, forming the mainstream smoke (MSS) aerosol, that consists of both gaseous and suspended particles in the form of liquid droplets, containing a wide variety of condensed organic compounds (Knishkowy and Amitai, 2005). Then, the smoke will bubble into the water jar, being cooled and diluted there, and finally a post-bubbling MSS is carried through the hose to the smoker (Knishkowy and Amitai, 2005).

Although a number of adverse health consequences have been epidemiologically associated with the use of narghile smoking as heart disease and oral cancer, other fields of research, especially histopathology is very limited (Akl *et al*., 2010). However, most of the corresponding studies were led in the past decades, and the authors of recent reviews have not realized that the products were either not detailed or not the one of growing concern (flavored moassal with a certain type of charcoal (quicklighting). This has resulted in a growing global confusion, including in meta-analysis (Chaouachi, 2011; Neergaard *et al*., 2007).

Hence, among the scarcity of histopathological research about narghile smoking, this study may increase the public concern about the narghile use, hoping it would help to uncover the negative face of this smoking method, through revealing the potential adverse effects of narghile smoke from two tobacco products, that differed principally in their components, on some histological parameters, that weren't highlighted by literature on selected tissues in an animal model (albino rat).

2. Materials and Methods

2.1. Experimental animals

Wistar albino male rats, *Rattus norvegicus*, with an average weight 215±2.5g were used. The animals were obtained from the University of Jordan colony and

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maintained under optimal conditions including diet and temperature.

2.2. Histological study design

This study is based on the chronic exposure of thirty experimental albino rats to the post-bubbling narghile MSS, coming from the complete heating/burning of 20 g from one of either two different narghile tobacco products: moassal or tumbak, for a period of 3 months, one session a day. An automated smoking machine as discussed in (Shraideh *et al.*, 2011) was used to expose the rats to narghile MSS. Each cycle of the smoking regimen lasts for 90 seconds and consists of three successive steps, operating as follows: Narghile smoke is drawn through the inhalation chamber continuously for 30 sec. An inlet to fresh air is then opened, allowing fresh air to be introduced instead of smoke, which will be washed out of the chamber. The washing out process will also take 30 sec. In the last 30 sec, the vacuum pump will be turned off, and rats will be allowed to breathe fresh air, normally (Shraideh *et al.*, 2011).

Narghile water was changed, and the tube was cleaned with distilled water after every experiment.

Albino rats were divided into 3 equal groups. The first group was the air-exposed control one, the second contains rats that have been exposed to moassal smoke, and the third group exposed to tumbak smoke. Following the exposure period, a histological examination by light microscopy of tissue pieces from the middle of tracheal segments and the anterior aspect of the right middle lobe of lung tissue was done. For each type of tobacco product, tissue pieces were taken from three randomly-selected albino rats.

2.2.1. Protocol of light microscopy

After overnight recovery from the last smoke exposure, rats were sacrificed by ether anaesthesia, and tissues of trachea and lung alveoli were gently dissected out, washed well with normal saline (0.9% NaCl), and fixed in 10% salined formalin. Using an automated tissue processor, tissues were fixed, dehydrated, cleared, and finally infiltrated by a hot liquid paraffin wax. To be ready for sectioning, tissues were embedded in paraffin. A ribbon of tissue sections were then obtained on a manual rotary microtome (Spencer 50) at 5 µm thickness. Two baths of 30% ethanol and a hot tap water were used to overcome the folding tendency. Thereafter, tissue sections were loaded on a glass slide meshed with egg albumin, dried, stained with classical haematoxylin and eosin stain (H&E), and finally mounted using Distyrene, Plasticizer, and Xylene (D.P.X.) mountant.

3. Results

3.1. Effect on the trachea

Control sections showed healthy ciliated pseudostratified columnar epithelium, mucosal and fibroelastic layers normally seen in tracheal tissue (Figure. 1).

3.2. Moassal smoke-exposed group

The tracheal mucosa of this group was adversely affected; showing an increase in the number of epithelial cells, amalgamation of cilia, presence of inclusion bodies, and lymphocytic infiltration (Figure. 2).

3.3. Tumbak smoke-exposed group

Profound epithelial cell proliferation and lymphocytic infiltration were observed in tracheal sections of this group. Cilia were either amalgamated or almost lost in other sections (Figure. 3).

3.4. Effect on alveoli of the lung

Photomicrographs of lung alveoli from control animals revealed the normal appearance of their characteristic simple squamous epithelium (Figure. 4).

3.5. Moassal smoke-exposed group

Lung alveoli of this group showed clear thickening in the connective tissue, and lymphocytic infiltration (Figure. 5).

3.6. Tumbak smoke-exposed group

Lung alveoli of this group showed areas with profound thickening in the connective tissue (Figure. 6).

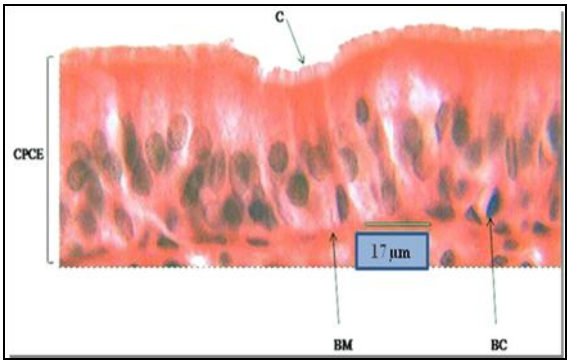

Figure 1. Section of normal tracheal tissue. CPCE: ciliated pseudostratified columnar epithelium, C: cilia, BM: basement membrane, BC: basal cell. H&E stain.

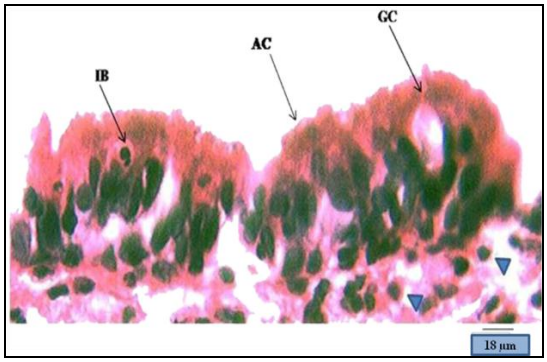

Figure 2. Section from the tracheal mucosa of moassal smoke-exposed rat, showing an increase in the number of epithelial cells, and lymphocytic infiltration (triangles). IB: inclusion body, AC: amalgamated cilia, GC: goblet cell. Triangles indicate lymphocytes. H&E stain.

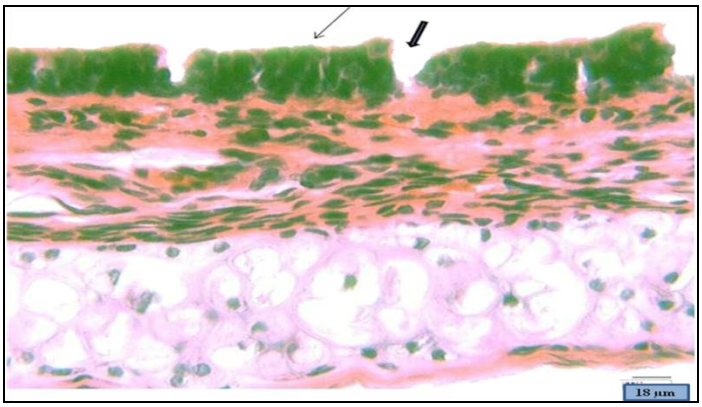

Figure 3. Trachea of tumbak smoke-exposed rat. The thick arrow indicates an area where the epithelium was disrupted. The thin arrow indicates a profound loss of the cilia. Epithelial cells are highly proliferated. H&E stain.

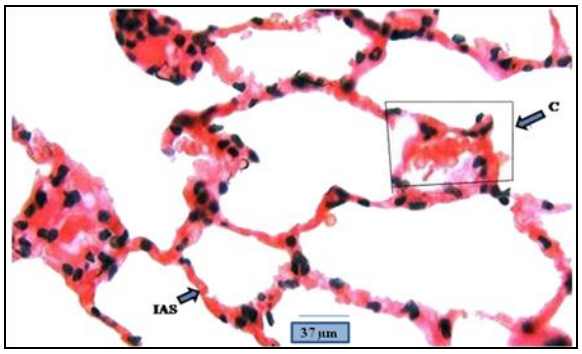

Figure 4. Control lung alveoli. IAS: interalveolar septum, C: capillary. H&E stain.

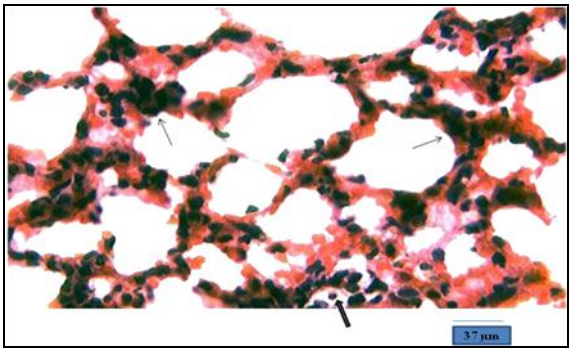

Figure 5. Lung alveoli of moassal smoke-exposed rat. Thin arrows denote areas with obvious alveolar wall thickening. The thick arrow indicates a lymphocytic cell. H&E stain.

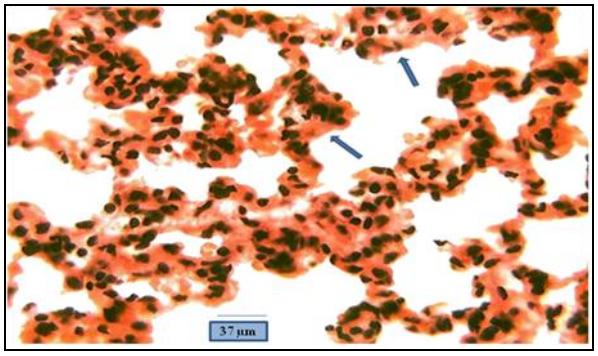

Figure 6 Lung alveoli of tumbak smoke-exposed rat. The arrows indicate obvious alveolar disruption. H&E stain.

4. Discussion

Cells of trachea and lung alveoli showed an adaptation by altering their pattern of growth, resulting in a hyperplasia. However, the limit of this adaptive response was exceeded, resulting in cell injury, which may be due to an oxidative stress; following the statement by (Ben Saad *et al*., 2010), that oxidative stress was increased significantly by regular water-pipe smoking, and the observation by (Sharma *et al*., 1997) of the presence of elevated levels of free radicals in peripheral blood neutrophils of water-pipe smokers. However, further support to the occurrence of oxidative stress associated with water-pipe smoking, which can lead to an imbalance in the production/ consumption level of reactive oxgyen species (ROS), would come from the following two studies: The first study, done by (Al-Numair *et al*., 2007),showed a significant increase in malondialdehyde -a biomarker for oxidative stress-, and a significant decrease in vitamin C - a potent antioxidant- in water-pipe smokers. The second study, done by) Wolfram *et al*., 2003), investigated the potential effect of smoking narghile on oxidation injury, by monitoring parameters of the (iso) eicosanoid system in narghile smokers. Two biomarkers of *in vivo* oxidative stress: 8-Epi-prostaglandin F2 alpha (8-epi-PGF2 alpha) and malondialdehyde were significantly increased after a single smoking session, and that repeated daily smoking induced a persistent long-lasting oxidation injury.Based on these facts, the following discussion is actually revealing the degree of cellular injury in two of the primary routes for smoke exposure (trachea and alveoli of the lung).

Trachea: The obvious disruption of the tracheal epithelium occurred by exposure to either moassal or tumbak smoke which caused powerful epithelial damage; will paralyze the cilia, enabling harmful foreign particles as dust or bacteria to remain in contact with the respiratory membranes for a prolonged periods, easily reach the lamina propria, where they can invade blood capillaries or lymphatic vessels, increasing the risk of toxic damage. Cracks that frequently observed within the tracheal epithelium are due to cell degeneration.

Ciliary amalgamation that can be viewed as part of epithelial disruption, may result from the hyperplasia of mucus-secreting submucosal glands, and may affect the airway clearance mechanisms. Inclusion bodies were observed, and they referred to any small amorphous blackish aggregate of smoke toxicants, primarily tar components.

The observed loss of cilia especially in tumbak-treated sections may be related to the high degree of nicotine it contains, through its effect on microtubules; polymerization / depolymerization of tubulin (Zenzes and Bielecki, 2004). Acetaldehyde and acrolein are suspected to play a role in the damage of cilia. Acetaldehyde was able to impair the ciliary function and beat frequency, by inhibiting ciliary dynein ATPase activity, and binding to ciliary proteins critical in the functioning of dynein and tubulin, whereas acrolein was found to adversely perturb the cilia by reducing its beat frequency, in cultured bovine bronchial epithelial cells (Dye and Adler, 1994).

Alveoli of the lung: Concerning the layers of cells lining the alveoli and the surrounding capillaries, are each only one cell thick, and are in very close contact with each other to facilitate gas diffusion between them; it is expected that the prominent thickening of the alveolar wall (pulmonary hyperplasia and hypertrophy) will compromise its capacity for gaseous exchange, resulting in a reduced gas transfer. According to the presence of extravasated erythrocytes, it could be simply justified by vascular injury. Lymphocytic infiltration may mediate the occurrence of inflammation. However, lymphocytes together with other inflammatory cells are frequently found in the bronchoalveolar lavage (BAL) of narghile smokers. For instance, Ourari, *et al*. (2006), have compared the cytology of the bronchoalveolar lavage BAL fluid (macrophages, lymphocytes, neutrophiles, and eosinophiles), and the lung function in 30 narghile users and 10 cigarette smokers. Researchers found that regular use of narghile induces a rise in the overall cell number in BAL. The increase does not seem to bring about significant changes in a number of lung function parameters when compared to cigarettes. The FEV1 and lung capacity were significantly higher. These results were also reported in the comprehensive critical review drawn by (Ben Saad *et al*., 2010).

5. Conclusion

No definitive conclusions can be drawn because, first, it is only an animal experimentation; second, it is based on exposing animals to smoke in a closed chamber. However, based on this experiment, smokers could be advised not to smoke on a daily basis and in poorly ventilated areas.

Regarding the smoking machine, we can suggest that the method of smoke exposure should be improved in a future study based on the same machine, but it could be set with different

parameters (for instance reducing puffing period, even if the animals may be exposed to a longer duration).

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Haematological Responses of Wild Nile Tilapia *Oreochromis niloticus* after Acclimation to Captivity

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Abstract

The effects of acclimation to captivity on blood composition of Nile tilapia *Oreochromis niloticus* were investigated. A total of ninety fish, comprising forty five each of juvenile (mean length 10.22cm ±0.26SD; mean weight 54.62g±6.74SD) and adult (mean length 19.4cm±7.21SD; mean weight 346.22g±2041SD) were harvested from a reservoir at African Regional Aquaculture Centre, Aluu, Port Harcourt and acclimated in the experimental tanks for a period of seven days. Before acclimation significant difference ($p < 0.05$) were observed between the blood parameters of adult and juvenile fish with the female consistently having higher values than the males in all the parameters. After acclimation to captivity, there was significant reduction ($p<0.05$) in the values of haemoglobin (Hb), packed cell volume (PCV), red blood cell (RBC), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), lymphocytes and thrombocyte, while the values of mean corpuscular volume (MCV), white blood cell (WBC), neutrophils (NEUT) and monocytes (MON) increased significantly ($p < 0.05$). Results from this study therefore suggest that sex and size have some degree of influence on the blood characteristics of *O. niloticus*.

Keywords: Haematology, tilapia, fish, acclimation, aquaculture.

1. Introduction

Fish represents at least 55% of the animals protein consumed in the diet of Nigerians (Anyanwu *et al.*, 2002), with fish demand outstripping the supply this has led pressures on the natural fisheries reserves, which have been subjected to gross over fishing for several years. It is therefore imperative in order to achieve self-sufficiency for the country (Adesulu, 2001).

The choice of species to culture and efficient management practices are very crucial to the overall success of any aquaculture venture, according to Gabriel *et al*. (2007a). Effective handling procedures of species in the culture medium are key practical factors which determine the profitability and sustainability of aquaculture as an enterprise. Effective management practice has been recognized as a key to profitable and sustainable fish farming in Nigeria. This has led to the application of various manipulation strategies to maximize fish production in the culture environment (Akinrotimi *et al*., 2007a). According to Akinrotimi *et al*. (2007b), one of the production procedures commonly used in aquaculture is acclimation, which is a preconditioning of fish before stocking in ponds and before use for experimental studies. Acclimation is, therefore, the modification of biological structures to minimize deviation from homeostasis, despite change in environmental factors (Gabriel *et al*., 2007b). It is a general practice to subject fish species to be used in laboratory experiment to a minimum acclimation period of seven days (Gabriel *et al*., 2004). It is believed that during this period the fish may show symptoms of hidden disease that may assist in the separation of apparently healthy fish for any trial or culture (Gabriel *et al*., 2007c).

Haematological variables have been used as indices of fish health status in a number of fish species to detect physiological changes as a result of stress condition such as exposure to pollutants, hypoxia, transportation, anaesthetic and acclimation (Akinrotimi *et al.*, 2009). Haematological indices are therefore ready tools used by fish biologists and researchers in many parts of the world. This is so because fish are closely associated with the aquatic environment and the blood will reveal conditions within the body of the fish long before there is any visible sign of disease (Fernades and Mazon, 2003).

Fish may be stressed when captured and when held in captivity. Effects of acclimation to captivity on haematological parameters of fish have been studied in a number of fish species, *Clarias gariepinus* (Ezeri *et al.*, 2004, Gabriel *et al*., 2004), *Sarotherodon melanotheron* (Akinrotimi, *et al.*, 2006; Gabriel *et al*, 2007d), *Tilapia guineensis* (Akinrotimi *et al*., 2010). There is limited information on the blood parameters of *O. niloticus*, which is a good experimental fish for studying the effect of environmental conditions on blood parameters (Omoregie

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and Oyebanji 2002). For this reason, the objective of this study was to assess the effects of acclimation to captivity on haematoligical characteristics of the species.

2. Materials and Methods

A total of 90 *O. niloticus* comprising 45 each adult (mean length 19.48cm±7.21SD; mean weight 246.22g ± 20.41SD) and juvenile (mean length 10.22cm ± 1.26SD; weight 54.62g ± 6.74SD) were harvested from reservoir in African Regional Aquaculture Centre (ARAC) Aluu, Port Harcourt, Rivers State, Nigeria. After harvesting, the fishes were then sorted into adult and juvenile sizes and blood was immediately taken from 10 fish in each sizes, with 21 gauge hypodemic needle and preserved in disodium salt of ethylene diamine tetra-acetic acid (EDTA) bottles for analysis.

The fish were then transferred into indoor hatchery, where they were acclimated in six rectangular tanks (0.6 x 0.6 x 1m^3). Half of the water in the experimental tanks was exchanged on daily basis. The fish were fed twice a day with pelleted feed (40% crude protein) at 3% body weight. Some water quality parameters were taken also on daily basis. Temperature was measured using mercury in glass thermeter ($^{\circ}$C), pH with pH meter (Model H1 9812, Hannah Products, Portugal). Salinity was determined by using hand held refractometer (Model HRN-2N Atago Product Japan). The dissolved oxygen (DO), nitrate and nitrate levels were determine using the methods described by APHA (1985).

After seven days, another set of blood samples, similar to the first, were collected again, and taken to laboratory for analysis. Standard haematological procedures described by Brown (1980) and Blaxhall and Daisley (1973) were employed in the assessment of haemoglobin, packed cell volume, red blood cell, lymphocytes, neutrophils, monocytes, platelets and erythrocyte sedimentation rate. While the values of mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH) and mean corpuscular haemoglobin concentration (MCHC) were calculated according to Miale (1982).

The data obtained were collated and analyzed with analysis of variance (ANOVA), where differences existed, mean separation was done with the Turkey HSD test at 0.05% probability (Wahua, 1999).

3. Results

The physico-chemical parameters monitored in the tanks during the trial were within the same range in both the reservoir and experimental tanks with the only difference ($p < 0.05$) recorded in the dissolved oxygen level (Table 1).

The blood characteristics of adult *O. noloticus* before and after acclimation for seven days indicated a significant reduction in the values of Hb, PCV, RBC, MCHC, Lymph and Platelets in both male and female fish after acclimation (Table 3). But the values of MCV, WBC, NEUT, MON and ESR increased in both sexes (Table 3). However, the value of MCH did not change in male fish but it increased significantly ($p < 0.05$) in female fish after acclimation (Table 2).

Acclimation to captivity caused a reduction ($p < 0.05$) in the blood characteristics of male and female juvenile *O. niloticus* (Table 3) of Hb, PCV, RBC, MCH, MCHC, Lymphocytes and Platelets. However, in both sexes the values of MCV, WBC, neutrophils, monocytes and ESR increased ($p < 0.05$). These changes were more pronounced in male fish than the female after acclimation. The pooled data of the blood characteristics of *O. niloticus* irrespective of life stage (Table 4) revealed that the values of al the blood variables were raised ($p<0.05$) after seven days acclimation.

Table 1. Physico-chemical variable of reservoir and acclimation tanks

Variables	Reservoir	Experimental Tank
Temperature (^{0}C)	29.30±1.26^a	28.78±3.14^0
Dissolved Oxygen (mg/l)	6.72±0.16^a	4.98±0.84^b
pH	6.58±0.28^a	6.51±0.38^a
Salinity(‰)	0.02±0.01^a	0.01±0.01^a
Nitrite (mg/l)	0.003±0.001^a	0.014±0.001^a
Nitrate	0.32±0.03^a	0.98±0.34^a

Means in the same row with different alphabets are significantly different numbers in brackets indicate the S.D, ($P \leq 0.05$).

Table 2. Blood characteristics of adult *O. niloticus* before and after acclimation for seven days (mean ± SD)

Variable	Male		Female	
	Before	**After**	**Before**	**After**
Hb	6.93±0.70[a]	3.99±0.19[b]	7.03±0.24[a]	4.93±1.25[b]
PCV	20.01±0.17[a]	17.68±0.80[b]	21.64±1.32[a]	17.21±0.81[b]
RBC	4.05±0.18[a]	2.27±0.49[b]	4.25±0.22[a]	2.05±47[b]
MCH	17.06±1.00[a]	17.94±0.08[b]	16.60±1.50[b]	23.91±0.45[a]
MCHC	34.62±3.23[a]	22.57±0.08[b]	32.62±3.25[b]	28.93±0.87[b]
MCV	49.42±1.87[b]	79.53±12.12[a]	50.91±0.42[b]	87.00±21.33[a]
WBC	21.73±0.44[b]	23.96±0.43[b]	23.01±0.39[b]	24.00±0.40[a]
Lymph	69.67±0.92[b]	56.95±5.83[b]	60.18±3.86[a]	56.95±5.83[b]
Neutr	36.94±1.06[a]	38.07±0.71[b]	36.42±3.59[a]	38.36±5.80[a]
Mono	3.37±0.14[b]	5.04±0.71[b]	36.42±3.59[a]	38.36±5.80[a]
Platelets	109.26±2.65[a]	100.26±0.80[b]	114.73±3.57[a]	102.75±3.49[b]
ESR	6.57±0.40[b]	9.36±0.27[b]	6.62±0.32[b]	10.42±0.3725[b]

Key: Hb – haemoglobin, (g/dl) PCV – Packed Cell Volume (%); RBC – Red Blood Cells (Cells x $10^{12/1}$); MCH – Mean Corpuscular Haemoglobin (pg); MCHC – Mean Corpuscular Haemoglobin Concentration (g/dl); MCV – Mean Corpuscular Volume (F1); WBC – White Blood Cells (Cells x $10^{0/1}$); LYMP – Lymphocytes (%); MONO – Monocytes (%); Plat – Platelets (cells x $10^{0/1}$); ESR – Erythrocyte Sedimentation Rate (mm/br).

Table 3. Blood characteristics of male and female Juvenile *O. niloticus* before and after acclimation for seven days (mean ± SD)

Variable	Male		Female	
	Before	**After**	**Before**	**After**
Hb	6.37±0.9a	5.04±0.06a	6.44±0.94	5.07±0.94a
PCV	20.01±0.17a	17.86±0.80b	21.64±1.32a	17.21±0.80b
RBC	2.68±0.13a	2.38±0.06b	3.09±0.08a	2.63±0.49a
MCH	23.87±4.57a	21.15±0.27a	20.81±2.49a	19.28±0.06a
MCHC	35.43±5.19a	30.82±0.88a	34.11±5.38a	30.66±6.62a
MCV	67.00±2.13a	68.66±2.91a	61.28±2.55a	67.53±14.41a
WBC	19.25±0.07b	22.04±0.72b	20.72±0.85b	23.43±0.71b
Lymph	63.74±1.16a	59.41±1.20b	64.86±1.27a	60.42±0.69b
Neutr	35.44±0.13B	36.44±0.99a	32.40±1.38b	35.84±0.85a
Mono	2.15±0.13b	4.13±0.21b	2.73±0.11b	3.73±0.16a
Platelets	92.60±7.10a	89.20±7.62a	103.46±1.96a	100.87±2.13a
ESR	6.57±0.40b	9.63±0.27a	6.34±0.19b	9.04±0.34a

Key: Hb – haemoglobin, PCV – Packed Cell Volume; ERS – Erythrocyte sedimentation rate; RBC – Red Blood Cells, MCH – Mean Corpuscular Haemoglobin; MCHC – Mean Corpuscular Haemoglobin Concentration; MCV – Mean Corpuscular Volume; WBC – White Blood Cells; Lymph – Lymphocytes; Neutr – Neutrophils, Mono – Monocytes; ESR – Erythrocyte sedimentation rate (Mean under each of the sexes with different alphabets are not significantly different at 0.05% ($p > 0.05$).

Table 4. Haematological characteristics of adult and juvenile *O. niloticus* irrespective of sex before and after acclimation for seven days

Variable	Male		Female	
	Before	**After**	**Before**	**After**
Hb	6.69±0.22a	4.76±0.22b	6.41±0.84b	5.05±0.59b
PCV	1965±0.24a	17.13±0.24b	18.47±0.56a	16.63±0.54b
RBC	3.520±0.15a	2.33±0.15b	2.88±0.24a	2.50±0.34b
MCH	19.58±0.92a	75.62±3.58b	22.34±3.69a	20.21±1.04b
MCHC	34.19±1.45a	20.57±0.92b	34.77±5.11a	30.74±4.22b
MCV	57.15±3.58a	75.62±3.58b	64.14±3.77a	68.09±9.31a
WBC	21.18±0.19b	23.36±0.19b	19.99±0.97a	22.73±0.19b
Lymph	62.11±1.00a	60.41±1.00b	64.30±1.25a	59.91±1.03b
Neutr	35.30±0.01b	70.39±0.50a	33.92±1.88a	36.14±0.89b
Mono	2.91±0.7a	4.40±0.75b	2.44±0.33a	3.93±0.22b
Platelets	105. 01 ±1.40a	98.27±1.40b	98.03±7.55a	95.73±8.12b
ESR	6.520±0.15a	9.33±0.13b	5.70±0.72a	8.65±0.75b

Mean in the same row with similar, with different superscripts are not significantly different at 0.05% ($p > 0.05$). Numbers in brackets indicate the S.D,

4. Discussion

Haematological parameters are routinely used for the evaluation of physiological environmental and husbandry stressors in fishes (Fanouraki, *et al*., 2007). In recent years good management practices have been advocated as effective ways of reducing stress in aquaculture (Gabriel, *et al*., 2007c). One of this method is acclimation, which is the sum total of the adjustment which fish makes to changes in the environment.. Hence, the mortality, recorded in this trial could be attributed to handling stress and sudden change in the environment particularly decline in the available dissolved oxygen. There were no significant differences between the physico-chemical parameters of the water (except DO) an indication that acclimation of *0. nitoticus* did not have an adverse effects on the other parameters as reported by Gabriel *et al*. (2004) who studied the effect of acclimation of *Clarias gariepinus* under similar conditions. The differences may be accounted for by the differences in the amount of water used in the previous study and mucus produced from the skin of the latter due to the stress of acclimation.

Several factors have been reported to affect haematological responses in fish. These include sex, age, size, environmental and physiological conditions (Sowunmi, 2003). Before acclimation the female consistently had higher blood values than the males corroborating the observation of Akinrotimi *et al.* (2007a) in *S. melanotheron*. This may be as a result of resting plasma cortisol level which is more in female than male fish (Akinrotimi *et al.*, 2010). After acclimation, the variation in blood parameters was more in male fish than in the female. It appears that the males are more responsive to the stress of acclimation than the females as reported by Gabriel *et al*. (2004) in *C. gariepinus*. Age and size of fish are crucial factors that affect fish response to stressors in aquaculture (Tavares - Dial, 2010). In this study changes in the blood parameters as a result of acclimation were more pronounced in adult compared to juveniles. This is comparable to the observations of Akinrotimi *et al*. (2010) in adult *T. guineensis*. This may be due to the size difference and hormonal interactions which are more in adult than the juvenile (Akinrotimi *et al.*, 2010b).

There was a significant reduction in the values of haemoglobin and red blood cells in both sizes of *0. niloticus* due to acclimation. Similar results were recorded by Gabriel *et al.* (2007a), in *S. melanotheron* subjected to acclimation for seven days. The significant reduction in these parameters is an indication of severe anaemia caused by acclimation in the exposed fish. The anaemic response could be as a result of disruption in erythrocyte production (Wintrobe, 1978; Omoregie, 1995), haemodilution (Sampath *et al*., 1993), and destruction of intestinal cells involved in the production of vitamin B12 used in the production of the haemoglobin portion of the red cells (Gardner and Yevich, 1970). The PCV was reduced ($P < 0.05$) in both sexes of juvenile and adult fish, a situation similar to that experienced by *T. guineensis* (Akinrotimi *et al*., 2010) under similar conditions and red porgy, *Pargus pargus* exposed to consecutive handling stress. (Fanouraki *et al.*, 2007). The low value of PCV in fish exposed to stress was attributed to a reduction in red blood cell volume caused by osmotic changes (Aiwan *et al*., 2009).

Changes in white blood cells and the differential counts neutrophils, lymphocytes and monocytes indicated a stress condition in *0. niloticus*. In *Limanda linianda* (Palsford *et al*, 1994) recorded similar increase in WBC which was believed to be caused by migration of white blood cells from the spleen to the blood circulation. Reduction of circulating lymphocytes caused by acclimation was also observed by in other teleost fishes like *Oncholiriynchus kitsutch* (McLeay, 1973), *Salmo trutta* (Espelids *et al*., 1996) and *Ictalurus punctuatus* (Ellsaesser and Clern, 1987). These were associated to re-trafficking of cells to lymphoid tissues which consequently leads to clearance of these cells from the blood stream (Harris and Bird, 2000). The increase in the neutrophils and monocytes values in acclimated experimental fish was similar to that reported by Omoregie and Oyebanji (2002), in the same fish species. This may be as a result of recruitment of more

cells to combat the effect of acclimation in an attempt to maintain external homeostasis.

Erythrocyte sedimentation rate (ESR), is a non-specific haematological parameter that may indicate the presence and intensity of disease state. The values are usually raised with increased tissue destruction as in acute infection and heavy metal poisoning among others (Blaxhall and Daisley, 1973). The mean corpuscular values are concerned with the volume of the average erythrocyte and the amount of. haemoglobin in the average erythrocytes. The values of this indices recorded in this study agrees with the report of Anyanwu *et al*. (2007) in *S. me1anotheron* transferred directly to fresh water.

5. Conclusions

The results of this study reveal that blood parameters of *O. niloticus* varied with age and sex. The haematological parameters of adult fish were generally higher than those of the juvenile; the values blood parameters in females were also higher than those of the males.

Acclimation to captivity caused a reduction in HB, PCV, RBC, MCHC, Lymph and platelets and increase in WBC, MCH, MCV, neutrophils, monocyte and ESR. These effects of acclimation to activity were more pronounced in male fish and in adult fish.

This information should be used in future acclimation experiments and the age as well as the sex of the experimental fish should be factors to be considered in experimental studies using *O. niloticus*.

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Quality Evaluation of Imported and Locally Produced Processed Cheese in Sudan

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Abstract

This study used sixty samples of locally and imported processed cheeses from the market in Khartoum North and Omdurman, Sudan. The chemical composition (protein content, fat content and ash content), titratable acidity and microbiological quality (total bacterial count, coliform count and yeast and mold count) of the processed cheese were estimated. The result indicated that there was a significant difference (p<0.01) in protein content, while there were non significant differences in fat content, titratable acidity and ash content between types of cheeses. Higher values were obtained for the protein (14.60%**)** and the fat content (32.41%**)** of the processed cheese samples collected during the year 2007 compared to those collected during 2008. Moreover, significant differences (p<0.05 and p<0.001) were obtained for protein and the ash contents of processed cheese samples respectively, which were collected during 2008. The titratable acidity revealed lower values for cheese samples collected during 2007 compared to those collected during 2008. Non significant differences in total bacterial, coliform and yeast and mold counts between types of cheese were obtained. The highest total bacterial and yeast and moulds counts were found in the processed cheese samples collected during 2008, whereas the high coliform count was reported for the cheese samples collected during 2007 and the lower count was estimated in the samples collected during 2008. Moreover, significant (p<0.001) variations were found in total bacterial count and yeast and mold counts of the cheese samples collected between and within the two years. The present study concluded that the processed cheese produced in Sudan is more or less similar to that imported from Egypt in chemical composition and microbiological quality. Hence this study encourages manufacturing processed cheese in Sudan in order to utilize huge amount of raw milk produced in the rural areas and also to minimize the cost of importation.

Keywords: Processed cheese, imported, locally produced, chemical composition, microbiological quality.

1. Introduction [*]

Research on technological processes on food is focused on two main goals: improving safety and quality of final products, and changing the characteristics of raw materials to obtain value-added products (Zamora *et al*., 2007). Processed cheese is obtained by mixing natural cheese and other ingredients, along with emulsifying salts, and using heat and agitation to produce a homogeneous product that is used in a variety of forms such as slices, blocks, shreds, and sauces (Kapoor *et al*., 2007). Process-induced modifications can have both beneficial and detrimental effects on technological aspects. In dairy processes, thermal treatment of milk aims at increasing shelf life and improving food safety of the final product (Zamora *et al*., 2007). Nour El Diam and El Zubeir (2006) reported that superior quality processed cheese can be produced if the milk is pasteurized before cheese processing to eliminate the original microflora of milk. The selection of base cheese is also critical in the manufacture of process cheese, as the base cheese serves to provide body and texture as well as flavor (Acharya and Mistry, 2005). Cheese pH influences almost all facets of cheese quality including flavor, texture, and appearance (Upreti and Metzger, 2007). Garimella *et al.* (2006) reported that the functional properties of process cheese are determined by the ingredients used in the formulation (i.e., type of natural cheese, age of natural cheese, amount of natural cheese, type and amount of emulsifying salt) as well as processing conditions (i.e., cooking temperature, cooking time and mixing speed during manufacture).

The presence of coliforms or yeasts is indicative of low processing temperature, especially at filling or negligent sanitation. The major microbiological problem with these products is growth of yeasts and molds, especially if free moisture is available at the surface (Marth and Steele, 2001). Moreover they added that some cheese defects may be caused by poor milk quality (late lactation milk, milk from mastitic animals, high in enzymes of animal origin, i.e. lipase and protease), inappropriate rate of acid development by the starter, or poor manufacturing and storage regimens.

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This study is intended to evaluate the chemical composition of imported (Egyptian) and locally processed cheese (Sudan). It is also aimed to compare their microbiological quality.

2. Material and Methods

2.1. Collection of cheese samples

Sixty samples of two types of processed cheese (Cheeke cheese made in Sudan and La vashe qui rit made in Egypt) were collected from the market in Khartoum State. The samples were collected during the period of March 2007 to June 2008.

2.2. Cheese samples analysis

The samples were analyzed for chemical composition (protein content, fat content and ash content, titratable acidity) and microbiological quality (total bacterial count, coliform count and yeast and mold count).

2.3. Chemical composition

The protein content was determined by Kjeldahal method and the fat content was determined by Gerber method, the ash content and titratable acidity were determined according to AOAC (2000).

2.4. Microbiological examination

Sterilization, examination of culture and preparation of the serial dilution from the samples and culturing methods were done according to Houghtby *et al*. (1992). Total bacterial count was determined using plate count agar (Biomark, B 298 – Biomark Laboratories,India) according to Houghtby *et al*. (1992). The plates were incubated at 32°C for 48 hours. Coliform count was determined according to Christen *et al*. (1992) using MacConkey agar (Central Drug House, New Delhi, 110002). The plates were incubated at 37°C for 24 hours. The count of yeast and mold were determined according to Frank *et al*. (1992) using potato dextrose agar ((M 096- Kalindi Industrial Corporation, Mumbai, 400056, India)). The plates were incubated at 25°C for 5 days. The growth was examined visually with naked eyes for colonies appearance and changes in media and then the colonies were counted using manual colony counter.

2.5. Statistical analysis

Statistical analysis was performed using the SAS (1997). Duncan multiple range test was used to determine the difference between means.

3. Results

Table 1 presents means, standard deviation, minimum and maximum values of chemical composition and microbiological quality of processed cheese.

Protein content showed significant differences ($P<0.01$) between types of cheese and it was high in locally produced cheese (14.02%) compared to the values recorded in imported cheese (13.28%). Data revealed non significance differences ($P>0.05$) between the types of cheese in fat content, titratable acidity and ash content (Table 2). However, fat content, titratable acidity and ash content were high for locally produced cheese (31.26%, 1.13% and 2.08%) compared to the values recorded for imported cheese (30.20%, 1.01% and 2.04%) as shown in Table 2.

The total bacterial count and coliform count were high in locally produced cheese (log 3.79 and log 3.34) compared to the imported one (log 3.67 and log 3.09), respectively (Table 2). However, the yeast and mold counts were found to be higher in imported (log 1.09) compared to the locally produced (log 0.94) cheeses. Moreover, non significant differences ($P>0.05$) were found between the sources in total bacterial count, coliform count and yeast and mold counts (Table 2).

Higher values were obtained for the protein (14.60%) and the fat content (32.41%) of the processed cheese samples collected during the year 2007 compared to those collected during 2008 (12.35% and 28.41, respectively). Moreover significant differences ($P<0.05$) were obtained for protein content of processed cheese samples collected during 2008 (Table 3). However the ash content (3.19%) was significantly ($P<0.001$) higher for cheese samples collected during 2008. The titratable acidity revealed lower values for cheese samples collected during 2007 compared to those collected during 2008 (Table 3).

The highest total bacterial and yeast and mold counts were found in the processed cheese samples collected during 2008 (log 3.61 and log 1.22, respectively) as shown in Table 3. The lowest values were estimated for the cheese samples obtained during 2008 and 2007 (log 3.19 and log 0.67, respectively), whereas the high coliform count (log 3.25) was reported for the cheese samples collected during 2007 and the lower count (log 2.51) was estimated in the samples collected during 2008 (Table 3). Moreover significant ($P<0.001$) variations were found in total bacterial count and yeast and mold count of the cheese samples collected between and within the two years (Table 3).

4. Discussion

It is clear from Table 2 that the processed cheese produced in Sudan is better in the compositional content compared to the imported Egyptian cheese. Appropriate selection of natural cheese is important to achieve a process cheese with the desired chemical and functional characteristics (Zehren and Nusbaum, 2000). They also added that research have highlighted some of the important physicochemical characteristics of a natural cheese that influence the functional properties of process cheese. It is important for process cheese manufacturers to be able to select a base cheese with the desired degree of proteolysis and according to commercial manufacturers (Acharya and Mistry, 2005). They added that natural cheese made from concentrated milk has also been found to influence the chemical as well as functional properties of process cheese.

There was a significant difference ($p<0.01$) in protein content between the examined types of cheeses; a significant ($p<0.05$) variation was also found between the years during which the cheeses were collected in protein content. This might be due to the variation of the storage time of the collected batches of the samples.

Table 1. Chemical content and microbiological quality of processed cheese in the markets of Khartoum State

Measurements	Mean ± SD	Minimum	Maximum
Protein (%)	13.65±1.51	8.90	15.90
Fat (%)	30.73±3.10	22.00	38.00
Acidity (%)	1.07±0.38	0.50	2.00
Ash (%)	2.06±0.70	0.32	3.90
Log total bacterial count (cfu\gm)	3.73± 3.88	2.21	4.68
Log coliform count (cfu\gm)	3.23± 3.36	1.85	4.12
Log yeast and mold (cfu\gm)	1.02± 0.97	0	1.6

Table 2. Difference between processed cheese types in chemical composition and microbiological quality.

Types of cheese	Protein (%)	Fat (%)	Acidity (%)	Ash (%)	Total bacterial count (log cfu)	Coliform count (log cfu)	Yeast and mold count (log cfu)
Imported	13.28[a]	30.20[a]	1.01[a]	2.04[a]	3.67[a]	3.09[a]	1.09[a]
Locally produced	14.02[b]	31.26[a]	1.13[a]	2.08[a]	3.79[a]	3.34[a]	0.94[a]

In this and the following Table: Means in the same column with a similar letter (s) are not significantly different at p= 0.05, according to Duncan Multiple range test

Table 3. Difference between years in the chemical composition and microbiological quality.

Years	Protein (%)	Fat (%)	Acidity (%)	Ash (%)	Total bacterial count (log/ cfu)	Coliform count (log/ cfu)	Yeast and mold count (log/ cfu)
2007	13.65[a,b]	31.75[a]	0.96[a]	1.44[c]	3.4[a]	2.82[a,b]	1.10[a]
	14.16[b]	30.58[a,b]	1.10[a]	2.00[b]	3.60[b]	3.25[a]	0.80[b]
	14.60[a]	32.41[a]	0.95[a]	1.80[b]	3.30[a]	2.9[a, b]	0.67[b]
2008	12.35[c]	28.41[b]	1.15[a]	3.19[a]	3.61[a]	2.95[a b]	0.83[b]
	13.48[a,b]	30.50[a,b]	1.19[a]	1.8[b]	3.19[a]	2.51[b]	1.22[a]

Hamed *et al*. (1997) and Nour El Diam and El Zubeir (2010) found that the protein content of processed cheese decreases by storage time, a result that might by due to limited degradation or assimilation of protein in cheese (Hamed *et al*., 1997). Kim *et al*. (1992) mentioned that the protein of cheddar cheese showed a tendency to increase during storage due to the rapid decrease in the moisture content. There were non significant differences between examined types of cheese in fat content, ash content and acidity in this study (Table 2 and Table 3). Similar findings were reported by Suleiman *et al.* (2011) who found that fat, protein, ash, and titratable acidity of cheese were not significantly affected by the time of sample collection. This indicated that the processed cheese is a stable product with a reasonable shelf life (Hanna and Nader, 1996). Schar and Bosset (2002) reported that processed cheese is often expected to be a stable product with a very long shelf life. Similarly Nour El Diam and El Zubeir (2007) reported that storage period was also improved after processing the Sudanese white cheese and gives chances of possibility of using processed cheese in Sudan. This might be because cooking process helps to destroy spoilage microorganisms and improve the shelf life of the process cheese (Siew *et al.*, 2004).

The microbiological picture as shown in Table 2 revealed that the locally produced cheese is more or less similar in the properties to the imported one. This suggested that it is reasonable to produce the processed cheese with an acceptable quality in Sudan, supporting the previous study that used Sudanese white cheese for production of the processed cheese (Nour El Diam and El Zubeir, 2007). They also reported that the huge quantities

of milk produced in the rural areas can be utilized by reprocessing cheese in towns to reasonable longer shelf life cheese (processed) from the Sudanese white cheese. Similarly, Hanna and Nader (1996) concluded that locally produced soft cheese in Iraq could be used instead of imported semi-hard cheese to make processed cheese of acceptable quality.

The high number of total bacterial count reported in the present study could be due to the high coliform count as shown in Tables 1, 2, and 3. The significant difference between coliform counts was in support to the findings of Suleiman *et al.* (2011). Massa *et al*. (1992) reported that high concentration of feacal coliforms was observed in 41 samples of Mozzarella cheese. Similarly Coveney *et al*. (1994) found that the incidence of coliforms were higher in soft, semi-soft and semi-hard cheese than in hard types. High coliform count in processed cheese might be due to poor processing conditions or post processing contamination (Nour El Diam and El Zubeir, 2006). Also the presence of coliforms or yeasts is indicative of low processing temperature, especially at filling or negligent sanitation. In addition to composition, pH and water activity, the presence of melting salts may be inhibitory to the growth of clostridia (Marth and Steele, 2001).

When comparing yeast and mold count of the processed cheese, it was found that there was non significant difference and this result is in agreement with the findings of Nour El Diam and El Zubeir (2006). This might be due to heat treatment that the processed cheese was subjected to during processing (Siew *et al.*, 2004). The count of yeast and molds of the processed cheese showed lower values compared to those from the Sudanese white cheese which indicated the improvement of the quality, which might be due to heat treatment (Nour El Diam and El Zubeir, 2006). Moreover molds are not supposed to grow on cheeses that are vacuum packaged, but they sometimes do as they tend to grow on cheese where pockets of air exist between the packaging material and cheese surface (Marth and Steele, 2001).

The results recorded on this study indicate that the locally processed cheese is better than the imported one concerning the compositional content, while the imported cheese revealed slightly better microbiological quality. Hence, it is recommended that the hygienic handling especially during processing, packaging and storage should be improved and controlled. In addition, official authorities should encourage the manufacture of processed cheese in Sudan in order to minimize the high cost of importation.

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Bioefficacy of Extracts of some Indigenous Nigerian Plants on the developmental stages of mosquito (*Anopheles gambiae*)

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Abstract

The bioactivity of hexane extract from the nuts of *Anacardium occidentale* (Linnaeus), ethanol extracts from the bark of *Myrianthus arboreus* (P. Beauv) and fruits of *Xylopia aethiopica* (Dunal), were studied at five concentration levels (0.1%, 0.2%, 0.3%, 0.4% and 0.5%) against the larvae, pupae and adults of *Anopheles gambiae* (Giles). Results indicated that *X. aethiopica* caused significantly ($P < 0.05$) higher mortality of larvae, pupae and adult mosquitoes than other plant extracts tested. It caused 100%, 57.50% and 92.50% larva, pupa and adult mortality, respectively at 0.5% concentration. Also, based on the lethal concentration average (LC_{50}) results, *X. aethiopica* was the most effective, with LC_{50} values of 0.23, 0.40 and 0.29 µg/ml on the larvae, pupae and adults *An. gambiae*, respectively, followed by *A. occidentale* (LC_{50} 0.28, 0.45 and 0.34 µg/ml), then *M. arboreus* (LC_{50} 0.32, 0.64 and 0.36 µg/ml). The results of our findings were discussed in line with use of biorationals as an affordable, readily accessible, and environmentally friendly alternative means of reducing malaria disease in Nigeria, by controlling *An. gambiae* mosquito, a major vector of malaria pathogen.

Keywords: *Anacardium occidentale*, *Anopheles gambiae*, fumigant toxicity, mosquito, *Myrianthus arboreus*, *Xylopia aethiopica*.

1. Introduction

Mosquitoes transmit diseases to more than 700 million people annually in Africa, South America, Central America, and much of Asia, with Africa being the most affected continent. Nigeria, being the most populous country in Africa, its citizens residing in the country, is at the greatest risk of malaria disease. Malaria is the most prevalent of mosquito borne diseases; being endemic in about 109 countries, affecting 190-330 million people and causing about one million deaths every year. *Anopheles* mosquito is the insect vector responsible for the transmission of the causative pathogen (plasmodium) of malaria (WHO, 1996; Akinkurolere and Zhang, 2007; WHO, 2010; RBM, 2011).

Over the last 50 years, insect pests control has mainly been with synthetic chemical insecticides such as organochlorines, organophosphates, carbamates, and pyrethoids, of which organophosphates and carbamates are the major classes in use today. However, problems due to pesticides resistance, negative effect on non-target organisms (including humans and the environment) (Rembold, 1984; FAO, 1992; Franzen, 1993) have been associated with use of synthetic chemical pesticides. In addition, these pesticides are expensive, more hazardous to handle, leave toxic residues in food products, and are not easily biodegradable. Thus, attention is fast shifting to alternative pest management strategies.

Before organochlorine and organophosphate insecticides were discovered in the late 1930s and early 1940s, botanical insecticides were important products for pest management even among the industrialized countries (Isman, 1997). Small holder farmers and researchers have often claimed successful use of plant products in insect pest control. Plant materials such as spices, vegetable oils, extracts, powders or ash (Ofuya, 1986; Ajayi *et al.*, 1987; Lale, 1992; Lajide *et al.*, 1998; Keita *et al.*, 2001; Akinkurolere *et al.*, 2006; Adedire *et al.*, 2011) have been reported for their insecticidal efficacy. And unlike synthetic chemical insecticides that kill both pests and non target organisms, natural insecticides including botanicals are relatively target specific (Isman, 1997). They are also biodegradable, environmentally friendly, and can also be used in insecticide resistance management programmes (Saxena, 1987). Hence, could serve as good alternatives to chemical insecticides.

WHO (2010) reported that the primary public health intervention for reducing malaria transmission at the community level is through vector control. It is the only intervention that can reduce malaria transmission from very high levels to close to zero. Therefore, in a resolve to align with the efforts of the federal government of Nigeria to eradicate malaria within its sovereignty, researchers have routinely screened botanicals for their potency against mosquitoes. The present study was therefore carried out to determine the bioactivity of three indigenous Nigerian plants namely: *Anacardium occidentale*

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(Linnaeus), *Myrianthus arboreus* (P. Beauv) and *Xylopia aethiopica* (Dunal) A. Rich against the larvae, pupae and adults of *Anopheles gambiae* (Giles). The toxicity of these plants against some insects had earlier been reported (Asawalam *et al.*, 2006; Oparaeke and Bunmi, 2006; Adedire *et al.*, 2011).

2. Materials and Methods

2.1. Collection of plant materials

The fruits of *X. aethiopica* and seeds of *A.occidentale* were purchased from Oba market, Akure, while the barks of *M. arboreus* were collected from the forest reserve at the Federal University of Technology, Akure (FUTA), Nigeria. The plant materials were identified in the department of Forestry and Wood Technology, FUTA.

2.2. Extraction of plant materials

The nuts of *A. occidentale* were sundried for three weeks to allow for easy cracking and to prevent the kernels from crushing. Thereafter, the nuts were mechanically cracked to obtain the kernel. Clean dried kernels were pulverized into fine powder using an electric blender (Binatone® Model BLG400) (Adedire *et al.*, 2011). Amount of 200 g was soaked in 2 litres of hexane and heated in water bath at 60°C for 1 h and then decanted. The solvent was separated from the extract by vacuum evaporation. The crude extract (semi-solid paste) was kept in a dark bottle, labeled and preserved in the refrigerator till further use.

The dried fruits of *X. aethiopica* were pulverized as described earlier. Amount of 200 g of the *X. aethiopica* powder was soaked in 100 ml of ethanol in a conical flask. The flask was capped with rubber cork and left for 24 h undisturbed. Afterward, the mixture was filtered with sterile filter paper (Whatman no. 1) into a fresh conical flask. The filtrate was transferred into the sample holder of the rotary evaporator where the ethanol solvent was evaporated at its boiling temperature of 70°C. The crude extract obtained was stored in the refrigerator (Aina *et al*., 2009).

The bark of *M. arboreus* was first chopped into small pieces before air-drying. When they were crisp dry, the bark was ground into powder with the aid of a blender as described above. Amount of 2 litres of ethanol was added to 1000 g of the plant powder and soaked for 48 h. Thereafter, it was filtered, and the filtrate was concentrated on rotary evaporator. The extract was also stored as above until further use.

2.3. Collection and rearing of mosquito

Mosquito baits, consisting of shallow containers with a large surface area, were established under a partial shade outside. The container was filled with rain water in order to mimic mosquito natural breeding environment and to attract adults for oviposition. Small quantity of industrial yeast was sprinkled on the surface of the water and allowed to decompose slowly; this was added to nourish the developing larvae. Wild mosquitoes were allowed to freely visit the bait and to lay eggs. Afterward, the containers bearing mosquito eggs/larvae were transferred to the laboratory, identified and maintained at temperature of 28 ± 2°C, 75 ± 5% RH and 14:10 L:D. Soon after the pupae emerged, they were transferred to a screened cage with dimension 20 × 20 × 20cm, where the adults emerged. After emergence, female mosquitoes obtained blood meal from caged immobilized albino rat; this is to make their eggs fertile, while male mosquitoes were fed on a 10% sucrose solution. Then egg-mass were kept to continue the next generation.

2.4. Effect of plant extracts on larvae and pupae of An. gambiae

Larvicidal and pupacidal activity of the plant extracts was carried out at different concentration by preparing the required stock solutions following the standard procedure (WHO, 1996). The desired concentrations were achieved by adding 1.0 µg of crude extract of any of the three plant materials to 100 ml of de-chlorinated water. From this, five concentrations (0.1%, 0.2%, 0.3%, 0.4% and 0.5%) of each plant extract were prepared. The extracts were mixed with water in a beaker at the desired concentration in the presence of small amount of yeast powder to serve as food source for the larvae. Then 10 larvae or pupae of *An. gambiae* were introduced into the beaker. Control (water only) beakers were similarly infested. There were three replicates for each concentration and the control. Mortality was observed over 24 h, after which the larva or pupae were introduced into distilled water to notice recovery. A recovery time of 5 minutes was allowed (WHO, 1996). The larval mortality in treatments was corrected for the controls (Abbott, 1925). Larvae or pupae were counted as dead when they were not coming to the surface for respiration and were insensitive to probe (Sivagnaname and Kalyanasundaram, 2004).

2.5. Fumigant effect of plant extracts on adult An. gambiae

The fumigant property of the plant extracts was used to assess their efficacy against the adults of *An. gambiae*. Ten adults were placed inside a test-tube and plugged with cotton wool. Strips of filter papers (3cm × 3cm) were soaked in varying concentrations of extracts and then suspended in the test-tube. Each treatment and the control were replicated three times. Mortality was recorded after 3 h of application.

2.6. Data analysis

For each treatment in 2.4 and 2.5 above, there were more than five trials from which data were pooled together and their averages (means) were determined. Data were subjected to analysis of variance (ANOVA), and means were separated using Tukey's test. The ANOVA and LD_{50} were performed with SPSS 11.0 software (SPSS, Inc., 2007). Because percentage mortality of *An. gambiae* in treatments was not normally distributed, data were first normalized by arcsine transformation before analysis. After analysis, back-transformed data were used in the tables.

3. Results

Tables 1 to 5 show the mean percentage mortality of larvae, pupa and adults of *An. gambiae* at 24 h after exposure to varying concentration levels (0.1%, 0.2%, 0.3%, 0.4%, and 0.5%) of *X. aethiopica*, *A. occidentale* and *M. arboreus* plant extracts. At the lowest concentration (0.1%) tested, plant extracts had significant

effect on larval ($F_{3, 8}$ = 1.56; P = 0.002) and adult ($F_{3, 8}$ = 21.88; P = 0.019) mortality, but not on pupal mortality ($F_{3, 8}$ = 15.63; P = 0.116). *X. aethiopica* caused significantly (P < 0.05) higher mortality of larvae and adult mosquitoes than other plant extracts tested (Table 1).

Table 1. Percentage mortality (mean ± standard error) of *Anopheles gambiae* at 24 h post treatment with 0.1% concentration of extracts of *Xylopia aethiopica*, *Anacardium occidentale* and *Myrianthus arboreus*

	Developmental stages		
Plant extracts	**Adults**	**Pupae**	**Larvae**
Control	0.00 ± 0.00 [a]	0.00 ± 0.00[a]	0.00 ± 0.00[a]
X. aethiopica	15.00 ± 0.20[c]	7.50 ± 0.20[a]	5.00 ± 0.37[b]
A. occidentale	7.50 ± 0.15[ab]	5.00 ± 0.15[a]	0.00 ± 0.00[a]
M. arboreus	2.50 ± 0.10[b]	0.00 ± 0.00[a]	0.00 ± 0.00[a]

Means within the same column followed by the same letter(s) are not significantly different at P>0.05 using Tukey's test.

At 0.2%concentation, plant extracts had significant effect on the mortality of larvae ($F_{3, 8}$ = 82.81; P = 0.002), pupae ($F_{3, 8}$ = 3.13; P < 0.001) and adults ($F_{3, 8}$ = 3.19; P < 0.001) of *An. gambiae* (Table 2).

Table 2. Percentage mortality (mean ± standard error) of *Anopheles gambiae* at 24 h post treatment with 0.2% concentration of extracts of *Xylopia aethiopica*, *Anacardium occidentale* and *Myrianthus arboreus*

	Developmental stages		
Plant extracts	**Adults**	**Pupae**	**Larvae**
Control	0.00 ± 0.00 [a]	0.00 ± 0.00[a]	0.00 ± 0.00[a]
X. aethiopica	27.50 ± 0.25 [c]	22.50 ± 0.25 [c]	40.00 ± 0.25 [b]
A. occidentale	22.50 ± 0.34 [b]	12.50 ± 0.34 [b]	37.50 ± 0.34 [b]
M. arboreus	2.50 ± 0.10 [a]	0.00 ± 0.00 [a]	30.00 ± 0.37 [b]

Means within the same column followed by the same letter(s) are not significantly different at P>0.05 using Tukey's test.

A similar trend of results was observed across the other plant extracts concentration further examined (Tables 3, 4 and 5). In all cases, larval mortality was the highest, while the lowest mortalities were observed in the pupa stages of the mosquito. And at 0.4% concentration, *X. aethiopica* evoked 100% mortality on *An. gambiae* larva (Table 4). The plant extract also caused 57.50% and 92.50% pupa and adult mortality, respectively at 0.5% concentration (Table 5). The efficacy of the plant extracts against *An. gambiae* was in the following order: *X. aethiopica* > *A. occidentale* > *M. arboreus*.

Table 3. Percentage mortality (mean ± standard error) of *Anopheles gambiae* at 24 h post treatment with 0.3% concentration of extracts of *Xylopia aethiopica*, *Anacardium occidentale* and *Myrianthus arboreus*

	Developmental stages		
Plant extracts	**Adults**	**Pupae**	**Larvae**
Control	0.00 ± 0.00 [a]	0.00 ± 0.00[a]	0.00 ± 0.00[a]
X. aethiopica	42.50 ± 0.49[c]	37.50 ± 0.49[c]	80.00 ± 0.49[c]
A. occidentale	37.50 ± 0.53[c]	30.00 ± 0.53[bc]	67.30 ± 0.53[bc]
M. arboreus	12.50 ± 0.57[b]	5.00 ± 0.57[ab]	42.50 ± 0.57[b]

Means within the same column followed by the same letter(s) are not significantly different at P>0.05 using Tukey's test.

Log probit analysis of the mortality data (Table 6) further revealed that *X. aethiopica* was the most effective with LC_{50} values of 0.23, 0.40 and 0.29 µg/ml on the larvae, pupae and adults of *An. gambiae*, respectively, followed by *A. occidentale* (LC_{50} 0.28, 0.45 and 0.34 µg/ml) then *M. arboreus* (LC_{50} 0.32, 0.64 and 0.36 µg/ml).

Table 4. Percentage mortality (mean ± standard error) of *Anopheles gambiae* at 24 h post treatment with 0.4% concentration of extracts of *Xylopia aethiopica*, *Anacardium occidentale* and *Myrianthus arboreus*

	Developmental stages		
Plant extracts	**Adults**	**Pupae**	**Larvae**
Control	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}
X. aethiopica	70.00 ± 0.47^{c}	52.50 ± 0.47^{d}	100.00 ± 0.00^{d}
A. occidentale	67.50 ± 0.67^{c}	33.00 ± 0.67^{c}	82.50 ± 0.00^{c}
M. arboreus	27.50 ± 0.73^{b}	15.50 ± 0.73^{b}	60.00 ± 0.73^{b}

Means within the same column followed by the same letter(s) are not significantly different at $P>0.05$ using Tukey's test.

Table 5. Percentage mortality (mean ± standard error) of *Anopheles gambiae* at 24 h post treatment with 0.5% concentration of extracts of *Xylopia aethiopica*, *Anacardium occidentale* and *Myrianthus arboreus*

	Developmental stages		
Plant extracts	**Adults**	**Pupae**	**Larvae**
Control	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}
X. aethiopica	92.50 ± 0.41^{d}	57.50 ± 0.41^{d}	100.00 ± 0.00^{c}
A. occidentale	75.30 ± 0.44^{c}	47.50 ± 0.44^{c}	100.00 ± 0.00^{c}
M. arboreus	52.50 ± 0.76^{b}	25.50 ± 0.76^{b}	92.50 ± 0.76^{b}

Means within the same column followed by the same letter(s) are not significantly different at P>0.05 using Tukey's test.

Table 6. LC_{50} (µg/ml) of *Xylopia aethiopica*, *Anacardium occidentale* and *Myrianthus arboreus* obtained from the mortality test on *Anopheles gambiae*

			Confidence limit			
Developmental stage	**Plants**	**LD_{50}**	**lower**	**upper**	**DF**	***P***
Larva	***X. aethiopica***	0.23	0.15	0.29	3	<0.0001
	A. occidentale	0.28	0.21	0.32	3	<0.0001
	M. arboreus	0.32	0.27	0.37	3	<0.0001
Pupa	***X. aethiopica***	0.40	0.34	0.46	3	<0.0001
	A. occidentale	0.45	0.39	0.48	3	<0.0001
	M. arboreus	0.64	0.59	0.72	3	<0.0001
Adults	***X. aethiopica***	0.29	0.25	0.37	3	<0.0001
	A. occidentale	0.34	0.28	0.43	3	<0.0001
	M. arboreus	0.36	0.32	0.45	3	<0.0001

4. Discussion

The toxicities of phytochemical compounds on target species vary depending on the plant part from which they have been extracted (Tuetun *et al.*, 2004). Other variations are due to responses of species and their developmental stages to the specific extract, solvent of extraction, geographical origin of the plant, phytosensitivity of compounds in the extract, effect on growth and reproduction, etc. (Jeyabalan *et al.*, 2003).

It has been widely reported that crude or partially purified plant extracts are less expensive and highly efficient for the control of mosquitoes, rather than the purified compounds (Cavalcanti *et al.*, 2004; Jenson *et al*., 2006).

All the plant extracts evaluated in this study effectively reduced the population of larvae, pupae, and adults of *An. gambiae* that were confined to simulated shallow water in the laboratory. The mortality effect, however, varied according to the plant and concentration of the extract.

The results of the present study show that *X. aethiopica* was the most potent of the three extracts. The markedly high toxicity of *X. aethiopica* against the larvae, pupae and adults of *An. gambiae* could be due to its strong pungent odour. A number of plants with high pungency have been reported for their bioactivity against insect pests (Dupriez and De Leener, 1998; Akinkurolere *et al*., 2006; Aina *et al*., 2009). The essential oil of *X. aethiopica* contains secondary plant compounds such as Alpha pinene, Betaphellandiene, Betapinene, 1- 8 Cineode, Gamma terpinene, Lindalyl acetate, Pinanol, Verbenene, Pinocarvone, L-carveol, Terpinene-4-ol, Myrtenal, Myrtenol, Cuminal, and Phellandral1 (Asawalam *et al.*, 2006). Since most of these compounds had been implicated in insect mortality through stomach poisoning,

contact toxicity etc., the high toxicity of *X. aethiopica* against the mosquito could therefore be due to the effect of one or more, or a combination of these compounds (Philipson and Wright, 1991).

A. occidentale and *M. arboreus* also exerted high toxicity against *An. gambiae*, though not as high as observed with *X. aethiopica* extracts. *A. occidentale* contain anacardic acid and cardinol (Rehm and Espig, 1991), quercetin and kaempferol glycosides (Oliver-Bever, 1986), triacylglycerols, fatty acids, several unsaponifiable compounds, triterpene, alcohols, sterols and tocopherols. The roots of *M. arboreus* contain several pentacyclic triterpenoids. Euscaphic acid, myrianthinic acid, tormentic acid, ursolic acid, a derivative of ursonic acid and triterpene acids have been isolated from stems. The bark contain tormentic and euscaphic acids, myriaboric acid, myrianthinic acid, and arboreic acid. The wood also contains myrianthiphyllin and lignin cinnamate (Burkill, 1985; Ngounou *et al.*, 1988; Tamboue Deffo and Nekam, 1993). These secondary plant compounds are similar to those found in *X. aethiopica*, some of which had been reported for their immunomodulatory, haemolytic, allelopathic and insecticidal activities (Echendu, 1991; Golob *et al.*, 1999; Adedire *et al.*, 2011).

The larvae of *An. gambiae* was the most susceptible developmental stage to plant extract treatment (with 100% mortality at 0.4% of *X. aethiopica* within 24 h), followed by the adults, then pupae. Other researchers have also made similar observations (Amusan and Okorie, 2002; Promsiri *et al*., 2006; Aina *et al.*, 2009). The mosquito larvae feed actively, by so doing, doses of plant active components could be ingested, thereby leading to stomach poisoning. Furthermore, *Anopheles* larvae lack a respiratory siphon, they breathe through spiracles located on the 8th abdominal segment and therefore must come to the surface frequently (Kaufmann and Briegel, 2004) to breathe. The plant extracts used in this study are oily; hence, the oils could block the spiracles, resulting in asphyxiation and death of the larvae (Akinkurolere *et al*., 2006; Adedire *et al.*, 2011). The pupae do not feed and are not active thus, reducing their chances of pesticide uptake cum susceptibility.

Results from this study are in accord with previous findings, where varying degrees of efficacy of plant materials against mosquito species were reported (Al Dakhil and Morsy, 1999; Amusan and Okorie, 2002; El-Bokl, 2003; Nathan *et al.*, 2005; Promsiri *et al*., 2006; Singh *et al.*, 2006).

Nigeria is located in malaria risk zones of sub Saharan Africa. And being a highly populated country, her populace are at the highest risk globally of deaths as a result of malaria (a mosquito borne disease), which kills more than one million people annually (WHO, 2010; RBM, 2011). Many of these deaths are as a result of poverty, because several poor Africans may not be able to afford the cost of medication or good accommodation to screen out mosquitoes. Therefore, in line with Nigeria governments' continued effort to eradicate malaria, researchers have routinely screened readily available plants for their cure for malaria or bioefficacy against the vector of malaria pathogen, the *Anopheles* mosquito. Here we conclude that the three plant materials used in this investigation, especially *X. aethiopica* was highly effective against *An. gambiae* and could be included in the management strategies of mosquitoes. In addition to their environmental friendliness, these plants are readily available, cheap, and would be affordable to the resource-poor persons in Nigeria.

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Quality Evaluation of Some Honey from the Central Region of Algeria

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Abstract

The quality of sixteen samples of *Apis mellifera* L. honey, from the center of Algeria, was evaluated by determining the physico-chemical characteristics. The following determinations were carried out: water content, total sugar, electrical conductivity, ash, pH, acidity (free, lactone, and total), hydroxylmethylfurfural (HMF) and color. The physicochemical parameters found are within acceptable ranges: water 13.36–17.93%, total sugar 80.17-84.73%, pH 3.58–4.72, total acidity 17.97-49.1 meq/kg, electrical conductivity 2.75×10^{-4} –7.19×10^{-4} S/cm, ash 0.075–0.33%, and color 4.1–9.2 Pfund index. The analysis of HMF showed that the majority of samples were exposed to a high temperature during processing or storage.

Keywords: Honey, quality, physicochemical parameters, Algeria.

1. Introduction

Honey is the natural sweet substance produced by *Apis mellifera* from the nectar of plants or from secretions of living parts of plants or excretions of plant-sucking insects on the living parts of plants, which the bees collect, transform by combining with specific substances of their own, deposit, dehydrate, store and leave in honeycombs to ripen and mature (Council Directive of the European Union, 2002).

The composition of honey depends on the plant species visited by the honeybees and the environmental processing and storage conditions (Bertoncelj *et al*., 2007; Guler *et al*., 2007; Sanz *et al*., 2004).

The carbohydrates are the major components of honey. The monosaccharides as fructose and glucose are the dominant fraction and occur for 85-95% of honey sugars. Honey also contains water and certain minor constituents such as proteins, enzymes, amino and organic acids, lipids, vitamins, volatile chemicals, phenolic acids, flavonoids, and carotenoid-like substances and minerals (Ball, 2007; Blasa *et al*., 2006). Blossom or nectar honey is derived from the nectaries of flowers and honeydew honey comes from the sugary excretion of some hemipterous insects on the host plant or from the exudates of the plants (Saxena *et al*., 2010).

Honey is generally evaluated by physico-chemical analysis of its constituents. The manipulation of honey and its possible adulteration is reflected in many of its physico-chemical properties such as HMF and sugar. Therefore, to ensure the authenticity, it is necessary to analyze honey samples in detail**.**

The studies of the physico-chemical properties of honey are important for the certification process that determines honey quality. The aim of this study was to evaluate and compare the quality of some honey samples from the central region of Algeria to the international standards.

2. Materials and Methods

2.1. Honey samples

Sixteen honey samples supplied by local producers from two geographical regions of center Algeria (Laghouat and Djelfa regions) have been studied. The samples were collected between 2009 and 2010, and stored at 4–6°C. Botanical classification was achieved when the pollen spectrum contained more than 45% of the corresponding dominant pollen (Louveaux *et al*., 1978). Pollen types were identified by comparing them with a reference collection of the Laboratory of analysis of the honey (Baba Ali, Algeria) (Table 1).

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Table 1. Honey samples and their botanical origin.

Samples	location	Botanical origin	Harvested period
L02	Kabegue	*Peganum harmala*	July 2009
L03	Khat alouad	Polyfloral	June 2009
L05	Hamda	Polyfloral	June 2009
L08	Tadjmout	*Trifolium sp.*	June 2009
L10	Taouyala	*Trifolium sp.*	June 2009
L11	Sidi makhlouf	Polyfloral	June 2009
L12	Gueltat sidi saad	Polyfloral	July 2010
L13	Kabegue	*Peganum harmala*	July 2010
D01	Megusem	Polyfloral	May 2009
D02	Megusem	*Ziziphus lotus*	July 2009
D05	Messad	Polyfloral	July 2009
D06	Messad	Polyfloral	July 2009
D07	Messad	Polyfloral	July 2009
D09	Ain oussara	Polyfloral	July 2010
D11	Medjbara	Polyfloral	July 2009
D12	Djalfa	Polyfloral	July 2009

(L: Laghouat region, D: Djelfa region).

2.2. Physico-chemical determinations

2.2.1. Sugar and water content

Sugar and water values were determined using a special refractometer reading at 20 °C (Carl-Zeiss Jena refractometer), with two direct reading displays, for the measurement of sugar content and moisture percent, respectively (AOAC, 1990). Sugar content was expressed as brix degrees.

2.2.2. Electrical conductivity

The Electrical Conductivity (EC) of a honey solution at 20% (dry matter basis) in CO_2-free deionized distilled water was measured at 20°C (AOAC, 1990) in a EUTECH instrument conductimeter (Con.520).

2.2.3. Ash content

The ash content was indirectly determined using the measured electrical conductivity and applying the following equation: X1= (X2 − 0.143)/1.743. Where: X1= ash value; X2= electrical conductivity in mS/cm at 20 °C (Piazza *et al*., 1991, Bogdanov *et al*., 1999).

2.2.4. pH, free, lactonic and total acidity

The pH was measured by pH-meter (WTW inoLab pH 750) in a solution containing 10g of honey in 75 mL of distilled water (AOAC, 1990).

The free, lactonic and total acidity were determined by the titrimetric method: the addition of 0.05 N NaOH, is stopped at pH 8.50 (free acidity), immediately a volume of 10 mL 0.05 N NaOH is added, and without delay, back-titrated with 0.05 M HCl from 10 mL to pH 8.30 (Lactonic acidity). Total acidity was obtained by adding free plus lactone acidities. Results were expressed as meq/kg **(**AOAC, 1990**)**.

2.2.5. Color measurement

The color was measured by the technique of visual Lovibond comparator (Series 2000, USA) (Aubert and Gonnet, 1983). The liquid honey (about 20g) was loaded into the measuring tube and the color compared with standards and the results obtained were expressed as "Pfund index".

2.2.6. Hydroxymethylfurfural analysis

Hydroxymethylfurfural (HMF) was determined by HPLC method (Fallico *et al*., 2004), Aliquots of honey samples were diluted to 50 ml with distilled water, filtered on 0.45 mm filter and injected into an HPLC (Varian 9012Q) equipped with a diode array detector (Varian, Star 330). The HPLC column was a Merck Lichrospher, RP-18, 5 mm, 125_4 mm, fitted with a guard cartridge packed with the same stationary phase (Merck, Milan). The HPLC conditions were the following: isocratic mobile phase, 90% water at 1% of acetic acid and 10% methanol; flow rate, 0.7 ml/min; injection volume, 20 ml. All the solvents were of HPLC grade (Merck, Milan). The wavelength range was 220–660 nm and the chromatograms were monitorated at 285 nm. HMF was identified from the peak in honey with a standard HMF (P>98% Sigma-Aldrich, Milan), and by comparison of the spectra of the HMF standard with that of one honey samples. The amount of HMF was determined using an external calibration curve, measuring the signal at λ=285 nm.

2.2.7. Statistical analysis

All results were statistically analyzed by one-way analysis of variance (ANOVA). Differences were considered significant for $p < 0.05$.

3. Results

Significantly, all parameters were very highly different among the samples ($p<0.001$). The results of physico-chemical parameters of honey samples from the two regions were presented in Table 2. The highest value for water content was found in the sample L10 (17,93%), this sample showed the lowest quantity of sugar. The total sugar contents ranged from 80.17 to 84.73%.

All studied honey samples were acidic in nature and the pH values varied between 3.58 and 4.72. Free, lactone and total acidity of

analyzed honey samples were between: 14.9-40.33, 3.06-8.98 and 17.97- 49.1 meq/kg, respectively.

The electrical conductivity was less than 8×10^{-4} S/cm. Medjbara honey (D11) has the highest conductivity (Table 2). The EC found in all the samples was typical for floral honey. The HMF content in five honey samples was lower than the allowed maximum limit of 40 mg/kg recommended by Codex Alimentarius (2001).

According to the method used by Aubert and Gonnet (1983), seven of our samples were light (≤ 6.2 Pfund index) and the remaining nine samples were dark (>6.2 Pfund index). L12 (9.2 Pfund index) is the darkest samples, followed by L05 and L10 with (8.3 Pfund index), the samples D11 (4.1 Pfund index) is the lightest samples, followed by L03 (5.1 Pfund index)

Table 2. Analysis of some physico-chemical parameters of *Apis mellifera* L. honey samples.

Samples code	Water content (%)	pH	Free acidity (meq/kg)	Lactone acidity (meq/kg)	Total Acidity (meq/kg)	HMF (mg/kg)	Electrical conductivity (10^{-4} S/cm)	Ash content (%)	Total sugar (%)	Color (Pfund index)
L02	14.3± 00	4.16±0.01	23.33±0.57	5.83±0.57	29.16±1.15	36.63±2.03	4.21±0.04	0.159±0.002	83.8±0.02	7.1
L03	17.36±0.25	3.58±00	40.33±0.28	8.76±0.25	49.1±0.52	49.41±0.54	3.91±0.02	0.142±0.001	80.95±0.22	5.1
L05	14.43±0.20	3.89±0.01	29.14±0.30	6.78±0.28	35.93±0.29	104.12±0.71	4.56±0.04	0.179±0.002	83.75±0.21	8.3
L08	13.96±0.20	4.08±0.02	23.98±0.49	5.16±0.37	29.15±0.14	116.61±3.67	3.72±0.02	0.131±0.001	84.15±0.13	7.1
L10	17.93±0.23	3.90±0.02	27.60±0.42	7.04±0.38	34.65±0.51	29.74±2.42	333±0.03	0.108±0.001	80±0.17	8.3
L11	16.53±0.05	3.69±0.03	25±0.62	5.64±0.57	30.65±0.25	72.86±1.84	4.65±0.02	0.184±0.001	81.76±0.05	6.2
L12	14.36±0.55	4.02±0.01	24.82±0.57	4.99±00	29.81±0.57	62.97±1.21	3.95±0.05	0.144±0.003	83.43±0,23	9.2
L13	14.16±0.11	4.43±0.01	17.22±0.25	6.82±0.28	24.04±0.38	18.21±0.52	5.22±0.01	0.217±0.001	83.93±0,11	8.3
D01	15.03±0.15	3.97±0.01	22.47±0.5	5.49±0.5	27.96±0.5	38.37±0.65	3.78±0.01	0.134±0.001	83.16±0.18	5.5
D02	16.33±0.15	4.29±0.00	19.72±0.25	5.13±0.31	24.85±0.15	85.07±1.64	5.30±0.04	0.222±0.002	81.95±0.13	7.1
D05	13.36±0.05	4.72±0.01	14.91±00	3.06±0.14	17.97±0.14	8.90±0.29	5.21±0.02	0.216±0.001	84.73±0.05	7.1
D06	14.13±0.05	3.91±0.02	25.42±0.49	7.06±0.37	32.48±0.51	100.29±2.04	3.66±0.02	0.127±0.001	84.01±0.05	8.3
D07	14.5±0.2	4.11±0.03	20.85±00	7.44±00	28.29±00	63.67±1.58	3.78±0.01	0.134±0.001	83.7±0.15	6.2
D09	17.53±0.11	3.6±0.01	40.08±0.28	8.98±0.25	49.06±0.14	205.04±3.9	3.93±0.02	0.143±0.001	80.76±0.11	5.5
D11	14±0.1	3.79±00	19.39±00	4.97±00	24.36±00	82.79±0.29	7.19±0.08	0.330±0.004	84.18±0.1	4.1
D12	15.16±0.11	3.91±0.02	16.33±0.85	4.37±0.51	20.70±0.86	138.77±2.61	2.75±0.03	0.075±0.001	83.05±13	5.5

4. Discussion

4.1. Water and sugar content

The water and sugar contents of honey are strictly correlated (Conti, 2000); the water content depends on various factors such as harvesting season, degree of maturity reached in the hive and climatic factors (Finola *et al*., 2007). All the values obtained were below 18% (Table 2) indicating a good degree of maturity are included in the water range limits approved by the European Commission (Council Directive of the European Union, 2002) and the Codex Alimentarius (Codex Alimentarius, 2001). Higher water content could lead to undesirable honey fermentation during storage (Saxena *et al*., 2010; Al *et al*., 2009). According to this result, lower water content is highly important for the shelf-life of the honey during storage. The honey samples having higher moisture content had lower total sugar and vice versa.

4.2. Electrical conductivity and ash content

The electrical conductivity of the honey is closely related to the concentration of mineral salts, organic acids and proteins; it is a parameter that shows great variability according to the floral origin and is considered one of the best parameters for differentiating between blossom honeys and honeydews (Mateo and Bosch-Reig, 1998; Terrab *et al*., 2002). The electrical conductivity of the samples varied between 2.75×10^{-4} –7.19×10^{-4} S/cm. According to Codex Alimentarius (Codex Alimentarius, 2001) and European Commission (Council Directive of the European Union, 2002) value for the nectar honey should be less than 8×10^{-4} S/cm (with few exceptions). Moreover, the values of EC of our samples are typical of blossom honeys. Besides this, the ash content of the present honey samples varied widely ranging from 0.075% to 0.33%. The observed ash contents were similar to Algerian honey samples (Ouchemoukh *et al.*, 2007). The maximum ash content was observed for the sample D11, followed by the sample D02 (0.222%). These differences in mineral content are dependent on the type of soil in which the original nectar bearing plant was located (Anklam, 1998).

4.3. pH and acidity

The pH of 15 honeys analyzed is less than 4.5; these are typical pHs in floral honeys. The pH values are of great importance during the extraction and storage of honey as they influence the texture, stability and shelf life (Terrab *et al*., 2003).

The acidity of honey developed due to the presence of organic acids. A high total acidity may mean that the

honey had fermented at some time, and that the resulting alcohol was converted into organic acid (Rodgers, 1979). In our samples, the values of total acidity ranged between 17.97–49.1 meq/kg; these values are similar to some locally produced honey in Algeria as reported by Chefrour *et al*. (2009). The total acidity was below the limit proved satisfactory in international trade (50 meq/kg of honey), indicating absence of undesirable fermentation in our samples.

4.4. Color

Based on the classification of Aubert and Gonnet (1983), nine honey samples are dark. The color of honey was related to its mineral content and the color of pollen (González-Miret *et al*., 2005; Terrab *et al*., 2004). On the other hand, the color intensity is supposed to be related to pigments (carotenoids, flavonoids, etc.), which are also known to have antioxidant properties (Frankel *et al*., 1998).

4.5. Hydroxymethylfurfural

HMF measurement is used to evaluate the quality of honey. It is not generally present in fresh honey (Zappalà *et al*., 2005). HMF values were found to be extremely high in 11 honey samples regarding the acceptable standard (≤40 mg/kg) (Council Directive of the European Union, 2002; Codex Alimentarius, 2001). Seven samples showed high levels of HMF (>80 mg/kg) and the most elevated HMF is observed in only one sample (205.04mg/kg). These excessive values of HMF indicate that there was overheating during processing, prolonged storage or adulteration with invert sugar (Singh *et al*., 1998; Kubis and Ingr, 1998; Doner, 1977; Zappalà *et al.*, 2005). Besides, honeys from subtropical countries have naturally high content of HMF due to the high temperatures (White, 1978). In our country without air-conditioning, storage temperatures during summer may reach somewhere close to 40°C.

5. Conclusion

The physico-chemical characteristics of five from the 16 honey samples analyzed in this study completely agree with the European Commission and the Codex Alimentarius indicating adequate processing, good maturity and freshness. Eleven honey samples did not agree with characteristics established in European and Codex standards relative to the HMF, although the other physico-chemical parameters were within the range of the allowable limits. The low moisture content helps to protect honey from microbiological activity and thus it can be preserved for long period.

This paper shows new results from honey composition of the central of Algeria (Laghoaut and Djelfa) that have not been studied yet. These results are also very important for the commercialization of the Algerian honey.

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Investigation of Genetic Diversity and Relationships among a Set of Rice Varieties in Iraq Using Random Amplified Polymorphic DNA (RAPD) Analysis

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Abstract

This study is an attempt to determine the genetic diversity and relationships among seven local rice varieties (*Oryza sativa* L.) and one commercial genotype by using the Random Amplified Polymorphic DNA (RAPD) technique. Seven universal primers used in this study produced (110) bands across eight varieties. Of these bands, 96 bands or 87.3% were polymorphic. The size of the amplified bands ranged between 190-3620 bp. The genetic polymorphism value of each primer was determined and ranged between 83-93%. In terms of unique banding patterns, the most characteristic banding pattern was for the Amber variety with primer A13 and for the Al-abasia variety with primer P07. Genetic distances ranged from 0.177 to 0.992 among rice varieties. Cluster analyses were performed to construct a dendrogram among studied rice varieties. The cluster analysis places most of the aromatic varieties into a close relation (subcluster) showing a high level of genetic relatedness and were distinct from non-aromatic (the other subcluster) with a few of independent varieties. Interestingly, a number of varieties originating from the same sources did form well defined groups, indicating association between the RAPD patterns and the geographic origin of the varieties. Therefore, cluster analysis grouped the eight varieties into three main clusters depending on their geographic origin; their ancestor and their aroma characteristics. The information generated from this study can be used in the future for rice breeding programs.

Keywords: Rice, DNA, RAPD, genetic distance, *Oryza sativa*.

1. Introduction

Rice (*Oryza sativa* L.) is one of the world's most important food crops, providing food for more than one third of the world's population. It is no longer a luxury food but has become the cereal that constitutes a major source of calories for the urban and the rural populations (Sasaki and Burr, 2000). Rice is grown in wide range of environments worldwide, even on a steep hill or mountain (Chakravarty, 1976). Most of the world's rice is grown and consumed in Asia, which constitutes more than half of the global population (Chakravarthy and Naravaneni, 2006). Iraq is one of the Asian countries which had suitable agro-climatic conditions for rice growing. Rice is the staple food for the greater majority of the Iraqi population. In Iraq, a number of traditional and improved varieties have been released for cultivation in different regions, such as the "Amber" variety, the most important traditional Iraqi rice variety (Chakravarty, 1976), and also "Furat" and "Yasmin", as introduced varieties.

There are numerous techniques available to investigate different genotypes of crop species and determine the purity of the variety to help in plant breeding programs, through improving the rice crop for the long-term and reducing of vulnerability of the crop. Prior to the availability of DNA-based markers, most genetic diversity studies in various crops were carried out using morphological and biochemical markers. Morphological and biochemical markers can be affected by environmental factors, growth practices and they are taking a long time to access, unlike DNA-based markers, which are not affected by environmental factors. Therefore, a DNA-based marker became one of these techniques which are very effective and reliable tools for measuring genetic diversity and relatedness among crop germplasm that was a major goal in evolutionary biology. The DNA based markers chosen for this study are Random Amplified Polymorphic DNA (RAPD). These markers were preferred because of the relative ease with which PCR assays can be carried out compared to other molecular markers. Advantages include rapid analysis, highly informative results, low cost and simplicity (Williams *et al.*, 1990). Single primers as short as decamers with random sequences are used to prime on both strands, producing a diverse array of PCR products (Sobral and Honeycutt, 1993). Prior knowledge about the genome is also not a prerequisite (Rabbani *et al.*, 2008; Shaptadvipa and Sarma, 2009), which makes RAPD a

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common method for such studies in different crops (Nair *et al.*, 2002; Gorji *et al.*, 2010; Szilagyi *et al.*, 2011).

Although there are a number of traditional and improved varieties of rice available in Iraq, no complete characterization or systematic analysis has been carried out so far into their genetic base and diversity, with the exception of one study by Al-Judy (2004) that studied other varieties using other universal primers. The aims of the current study are (i) identification and differentiation of various Iraqi rice varieties by generating a DNA fingerprint for each variety, (ii) estimation of the genetic diversity and determination of the genetic relationship among studied varieties by using RAPD markers.

2. Materials and Methods

2.1. Plant material

Eight rice varieties *(Oryza sativa)* were used in this study. Among the rice varieties used, one is a local variety, six are local varieties introduced from different regions (these seven varieties represent the major rice varieties currently grow in central and south regions of rice cultivation areas of Iraq), while one commercial genotype (Indian genotype) from the market was included for comparison. A detailed description of the plant materials used in the present investigation is given in table (1).

2.2. Genomic DNA Isolation

Total genomic DNA of all the studied varieties was extracted from dry seed using a commercial kit, High Pure GMO Sample Preparation Kit (Roche – Germany), to produce a rapid extraction and high quality extracted DNA. Purity and concentration of DNA was measured by spectrophotometer (Sambrook *et al.*, 1989). Genomic DNA integrity was detected by running on 0.8% agarose gel electrophoresis followed by staining with ethidium bromide and visualized under UV light (Maniatis *et al.*, 1982). DNA samples were diluted to a working concentration of 50 ng/µl in order to be use in the RAPD-PCR experiments.

2.3. Primer selection and RAPD assay

To identify primers promising detectable polymorphisms among rice varieties, nine decamers of oligonucleotides (Alpha DNA-Canada) were tested. After an initial screening, the primers were classified into two groups according to results obtained. The first group gave no amplified products and this group included (R01 and R03). The second group gave results in term of amplification and polymorphism, including (A07, A13, C05, D20, P06, P07 and R02). Only primers that had been earlier found to be polymorphic among rice varieties were used in this study.

Amplification reactions were performed in a volume of 25µl containing 12.5µl of Go Taq®Green Master Mix (Promega-USA), with concentration (1X) containing (10mM Tris-HCl (pH 8.3), 50mM KCl, 1.5mM $MgCl_2$, 200µM each deoxynucleotide triphosphate (dNTP) and 1 unit DNA polymerase), 10pmol of the primer, and 100 ng of template DNA. Amplification was carried out using a Mastercycler (Eppendorf-Germany), using the following program:- 1 cycle of 5 min at 94°C for initial strand separation, followed by 45 cycles of 1 min at 94°C for denaturation, 1 min at 36°C for annealing and 2 min at 72°C for primer extension. Finally, 1 cycle of 10 min at 72°C was used for the final extension, followed by a hold at 4°C (Rabbani *et al.*, 2008). Each PCR amplification reaction was repeated twice to ensure reproducibility.

Twenty microliter of PCR products were analyzed by electrophoresis in a 1.2% agarose gels at 5 Volt/cm for 2 hour in 0.5xTBE (10mM Tris-Borate, 1 mM EDTA) buffer, agarose gels were stained with ethidium bromide 0.5 µg/ml for 20-30 minutes. The 1 kb DNA ladder (250-10,000) bp (Promega-USA) was used as a molecular size marker. After electrophoresis, images of gels were captured using Gel Documentation System (Consort - Belgium).

2.4. Data analysis

A- Molecular Weight Estimation

Molecular weight was calculated by using the computer software M.W. detection program, Photo-Capture M.W. program from Consort, based on comparing the RAPD-PCR products with the known size of DNA fragments of a 1Kb DNA ladder (which consist of 14 bands from 250 to 10,000 bp).

B- Estimation of Genetic Distances

Only data generated from the detection of polymorphic fragments were analyzed. The amplification profile of all the used varieties for any given primer were compared with each other, the presence of band scored as "1" and the absence of the same band of the same size in other varieties scored as "0". The intensity of the bands was not taken into account. Only clear and reproducible amplified fragments were considered for genetic relationship analysis. Estimates of genetic distance (G.D) were calculated between all pairs of the varieties according to Nei and Li (1979) based on following formula:

$$G.D = 1-\{2Nab/(Na + Nb)\}$$

Where Na = the total number of fragments detected in individual 'a'; Nb = the total number of fragments shown by individual 'b' and Nab = the number of fragments shared by individuals 'a' and 'b'.

Cluster analysis was performed to construct genetic relationship tree diagrams among studied rice varieties using an Unweighted Pair-Group Method with Arithmetic Average (UPGMA). All computations were carried out using the Numerical Taxonomy and Multivariate Analysis System (NTSYS-pc), Version 1.7 package (Rohlf, 1993). The percentage of polymorphic bands was defined as ratio of the number of polymorphic bands amplified by a single primer to that of the total number of bands produced by the same primer.

3. Results and Discussion

3.1. DNA amplification and variety identification

The genetic diversity and the relationships among rice varieties were evaluated using RAPD markers amplified from nine universal primers. Among the nine decamer primers, there were two primers (R01 and R03) that give no PCR products; hence, they were eliminated from the

Table 1. Local and improved rice varieties used in the study.

Varieties' name	Pedigree	Varietal group	Breeding Institute
1.*Amber	Local (Iraqi)	Aromatic	AMRRS & SBAR
2.*Furat	Introduced from (Vietnam) in 1996	Aromatic	AMRRS & SBAR
3.*Yasmin	Introduced from (Vietnam) in 1998	Aromatic	AMRRS & SBAR
4.*Mashkhab-	Introduced from IRRI (Philippines) in 1978	Non-Aromatic	AMRRS & SBAR
5.*Mashkhab-	Introduced from IRRI (Philippines) in 1987	Non-Aromatic	AMRRS & SBAR
6.*Brnamge -4-	Introduced from IRRI (Philippines) in 2001	Non-Aromatic	AMRRS & SBAR
7.AL-abasia**	Radiation grain of Mashkhab -1- by Gamma ray	Non-Aromatic	SBSTC
8.Daawat	Commercial	Aromatic	Market

* SBAR : State Board for Agricultural Research, and AMRRS: AL-Mashkhab Rice Research Station.

**SBSTC: State Board of Seeds Testing and Certificatio

analysis. Each of the remaining seven primers varied greatly in their ability to resolve variability among varieties. Some primers generated several bands, while others generated only a few bands.

A total of 110 useful bands were scored from the amplification products with the seven random primers of DNA from eight rice varieties (Table 2). The number of amplification products generated by each primer varied from 8 (R02) to 24 (C05) and ranged in size from 190 bp (C05) to 3620 bp (P07). In general, sufficient polymorphism existed to allow distinction between the varieties tested with, polymorphism ranged between (83-93%), primer P06 produced the highest percent of polymorphism compared with primer C05. Of these 110 PCR products generated, 12.7% (14 bands) were monomorphic across all varieties. Many bands appeared in most of the varieties and were absent in only a few varieties.

The remaining 96 bands (87.3% of the total products scored) were polymorphic among the studied varieties; this was a relatively high level of the percentage of polymorphic bands obtained by random primers compared to reports of other RAPD studies in rice which were 77.4% (Nadarajan *et al.*, 1999) and 78.46% (Kumar *et al.*, 2010), while this percentage was comparatively similar to other rice studies at 89% (Rabbani *et al.*, 2008).

A total of 96 (87.3%) polymorphic bands were observed, ranging from 7 (R02) to 20 (C05) bands with an average of (13.7) polymorphic bands per primer across all the eight rice varieties. This average was similar to that observed in other rice studies using RAPD markers with Indian scented basmati and Italian rice cultivars. These reports observed that the average number of polymorphic bands per primer were 13.7 and 14.0 (Raghunathachari *et al.*, 2000; Porreca *et al.*, 2001), respectively. The average number of polymorphic bands was relatively higher than earlier reports, with an average of 2.7, 4.4, 5.4 and 6.6 polymorphic bands per primer (Virk *et al.*, 1995; Parsons *et al.*, 1999; Choudhury *et al.*, 2001; Skaria *et al.*, 2011). This discrepancy may relate to varieties and the selection of RAPD primers with scorable bands or the use of more diverse varieties. The arbitrary primer A13 was useful for discriminating varieties of distinct characteristics (Figure 1).

Some varieties could be distinguished from all other varieties with a selection of these primers. For instance, Amber and Daawat gave specific banding patterns with primers A13, D20, P07 and R02, and Al-abasia gave unique banding patterns with primers P07 and R02; while Furat gave unique banding patterns with primers C05 and P06. In most cases, the varieties of Yasmin and Furat, Mashkhab-1- and Mashkhab-2-, and also Brnamge-4- and Al-abasia were genetically related, although clear differences between them could be seen.

3.2. Genetic Distances

The ratio of genetic similarity among the eight varieties ranged from 0.007 to 0.822 (Table 3). The highest similarity (0.822) 82% was obtained between 'Furat' and 'Yasmin'. This was followed by (0.75) 75% similarity

between a pair of 'Mashkhab-1-' and 'Mashkhab-2-'. The lowest level of similarity (0.007) 0% was obtained between 'Amber' and 'Dawaat'.

In this study, the highest value of genetic similarity is relatively low when compared to the reports of other RAPD studies that obtained 0.50 to 0.96% genetic similarity among traditional and improved cultivars of Pakistani rice (Rabbani *et al.*, 2008), 49-89% genetic similarity among other Iraqi rice varieties (Al-Judy, 2004), and the Jaccard's similarity coefficient values ranged from 0.29 to 1.00% among the traditional medicinal rice cultivar of Kerala (Kumar *et al.*, 2010). However, similar values of similarity coefficients were obtained 0.83% among Indian elite rice varieties (Davierwala *et al.*, 2000) indicating a narrower genetic base in the improved varieties. Likewise, similarity coefficients ranging from 25 to 77.5% were observed among scented rice cultivars from India (Raghunathachari *et al.*, 2000).

3.3. Cluster analysis

Dendrogram was constructed based on Nei and Li's (1979) genetic distance using UPGMA cluster analysis and depicted genetic relationships among eight rice varieties, showing three major clusters I, II and III (Figure 2). As expected all introduced varieties: Yasmin, Furat, Mashkhab-1-, Mashkhab-2- and Brnamge-4-, were grouped into a cluster, including two subclusters, and other varieties Amber and Daawat into other two clusters. The first group, Yasmin and Furat formed one subcluster with the highest genetic similarity 82%. These were introduced from Vietnam and are aromatic varieties.

Table 2. Primers used for generating RAPD in traditional and improved varieties of rice from Iraq.

No.	Primer	Sequence (5'~3')	Total number of main bands	Number of polymorphic bands	Polymorphism %
1	A07	-GAAACGGGTG-	15	13	87
2	A13	-CAGCACCCAC-	15	13	87
3	C05	-GATGACCGCC-	24	20	83
4	D20	-ACCCGGTCAC-	20	18	90
5	P06	-TCGGCGGTTC	14	13	93
6	P07	-CTGCATCGTG-	14	12	86
7	R02	-GTCCTCGTGT-	8	7	88
8	R01	-CACACCGTGT-	---------	----------	----------
9	R03	-ACGGTTCCAC-	---------	----------	----------
	Total		**110**	**96**	

Table 3. Genetic distances among rice varieties based on the RAPD data.

Rice Varieties	Amber	Furat	Yasmin	Mashkhab-1-	Mashkhab-2-	Brnamge-4-	AL-abasia	Daawat
Amber	0.00000							
Furat	0.60391	0.00000						
Yasmin	0.47724	0.17784	0.00000					
Mashkhab-1-	0.52110	0.38819	0.27233	0.00000				
Mashkhab-2-	0.49471	0.42286	0.32547	0.24508	0.00000			
Brnamge-4-	0.56818	0.36115	0.33626	0.40510	0.31114	0.00000		
AL-abasia	0.71233	0.52131	0.50801	0.45009	0.52131	0.45383	0.00000	
Daawat	0.99257	0.64096	0.77022	0.75487	0.68018	0.68527	0.57503	0.00000

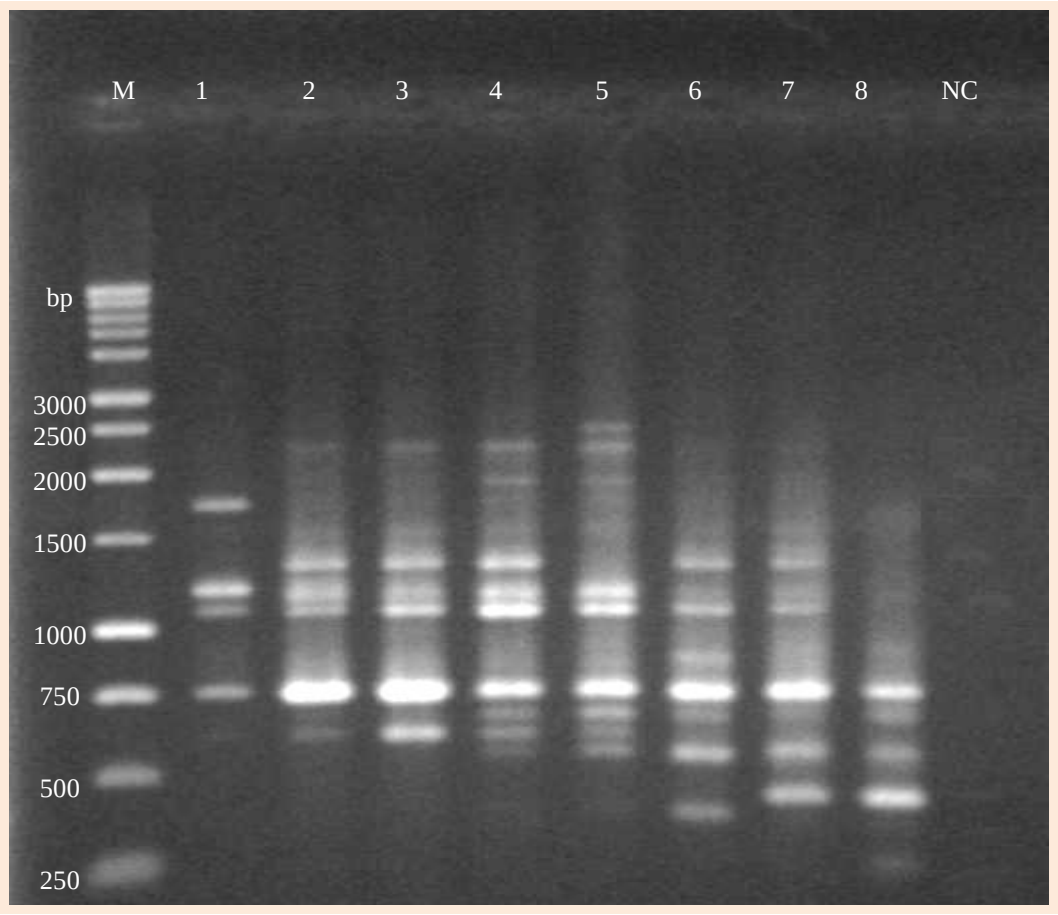

Figure 1. Agarose gel electrophoresis of a RAPD-PCR reaction for random primer A13 for DNA samples of the rice plants (under optimal conditions). Bands were fractionated by electrophoresis on a 1.2% agarose gel (2hr, 5V/cm, 0.5XTris-borate buffer) and visualized by ethidium bromide staining. M:1Kb ladder. Lanes: 1.Amber, 2.Furat, 3.Yasmin, 4.Mashkhab-1-, 5.Mashkhab-2-, 6.Brnamge-4-, 7.AL-abasia and 8.Daawat. NC: Negative control.

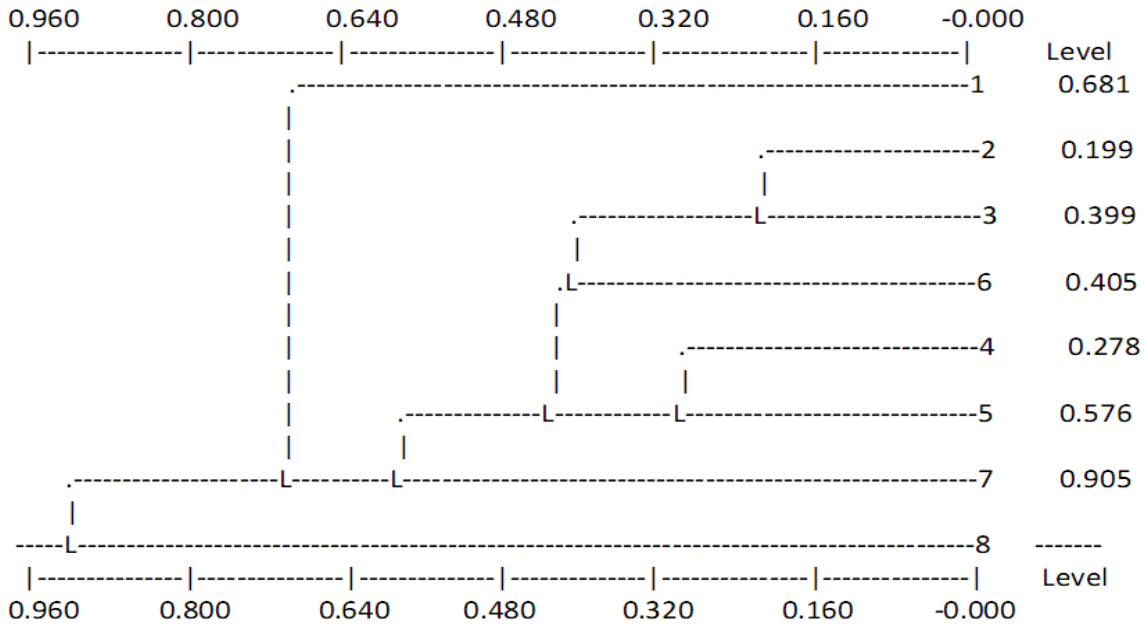

Figure 2. Dendrogram of rice varieties showing genetic distances based on RAPD data by using UPGMA cluster analysis. 1.Amber, 2.Furat, 3.Yasmin, 4.Mashkhab-1-, 5.Mashkhab-2-, 6.Brnamge-4-, 7.AL-abasia and 8.Daawat.

Mashkhab-1- and Mashkhab-2- formed another subcluster. These varieties were introduced from IRRI (Philippines) and are non-aromatic varieties. The Brnamge-4- variety was added to both subclusters. Though the variety Al-abasia was improved by radiating the grain of Mashkhab-1- with Gamma rays, it clustered with the IRRI varieties. In this analysis, the last Iraqi rice variety, Amber, appeared to be genetically distinct due to the lower level of the similarity with all other varieties. Therefore, it formed a separate group (II group). Likewise, the commercial variety 'Daawat' did not fall into any groups of Iraqi rice and it exhibited the lowest similarity with all varieties and also formed a separate group (III group).

Cluster analysis has placed most of the aromatic varieties together, showing a high level of genetic relatedness and these were distinct from those of non-aromatic varieties. Also the dendrogram indicates a clear pattern of division among the rice varieties based on geographic origin of the varieties. Therefore, cluster analysis grouped the eight varieties into three main clusters which correlated with their geographic origin; their ancestor and their characteristics. Similar results were reported in the study previously conducted using RAPD markers, where grouped rice varieties into different main groups depending on their geographic origin and their ancestor (Nadarajan *et al.*, 1999; Al-Judy, 2004; Skaria *et al.*, 2011) as also reported in other crops (Zhang *et al.*, 2005; Raju *et al.*, 2009; Al-Rawashdeh and Al-Rawashdeh, 2011; Kanbar and Kondo, 2011). The analysis clearly distinguished among studied rice varieties. Such studies can be used to study genetic differences of varieties for their identification. Therefore, it might be predicted that RAPD may be effective in analyzing polymorphism at the subspecies level in genus *Oryza*. In the present study RAPD markers provided sufficient resolution to distinguish closely related varieties.

The RAPD method may contribute to maximize the selection of diverse parent variety and to broaden the germplasm base in the future of rice breeding programs. The information generated from this study gives a clearer picture of their genetic relationship and might possibly be developed into a standard classification procedure in the future and will be used in identifying efficient strategies for the sustainable management of the genetic resources of rice crop.

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Study on Protease from Barley Tempeh and *in vitro* Protein Digestibility

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Abstract

In the present study, koji fermentation of barley seeds with *Rhizopous oligosporous* G-10 strain has a significant effect on the proteolytic activity and in vitro protein digestibility. The various process parameters were evaluated for the 100gm barley grains tempeh production, which was soaked for 8 hour in 500ml distilled water followed by boiling for 15 min in the presence of 1% NaCl and 1%glacial acetic acid and drying at 100 ^{o}C for 5 min. After pretreatment, barley grains were inoculated with 1% (v/w) spore suspension containing 10^{7} /mL and incubated at 30 ^{o}C for 36 hours. The maximum protease units (99.52 + 1.12 IU/g) were observed in fermented barley. The protein digestibility of 62% and 28% was found in fermented and unfermented barley during in vitro study.

Keywords: Koji fermentation, proteolytic, Rhizopous sp, protein digestibility.

1. Introduction

A fermented food from plant or animal origin are a part of the diet of the people in all over the world and is now oriented to develop low cost protein foods of plant origin to fulfill the protein (Danish *et al*., 2009; Mohammad *et al*., 2007). The second most abundant cereal grain in world is barley, consisting of 49-66% starch, 14-28 % dietary fiber and 9-22% crude protein, varies for different varieties (Kuswanto K and Rahayu, 2004). The preparation of many indigenous or traditional fermented foods remains today a household art for the improvement of nutritional value of the food through fermentation (Oscarsson *et al*., 1996). Microbial fermentation is considered as one of the oldest and most economical methods for food production and preservation. The fermentation process of cereals and legumes may increase the digestibility and bioavailability of proteins, carbohydrates, lipids, minerals and enhance the nutritional value such as vitamin content; shorten the cooking time and increase the microbial safety (McKeown N M, 2004). Therefore consumption of fermented food would lower food costs and promote better health (Behall *et al*., 2004a). Barely tempeh has been fermented through a process similar to soybean tempeh with Rhizopus spp, which is highly active proteolytic and lipolytic enzymes was suitable for producing tempeh from cereal grains (wheat, barley, oat, rye and rice) (Granfeldt *et al*., 1994). This early attempt to produce barley tempeh used dehulled andcracked barley (Hesseltine *et al*., 1967). However, commercial barley tempeh has not yet been produced. Previously, a patented barley tempeh procedure has been developed by fermentating whole pearled barley kernels (Gourmet korn) with selected strains of *R. oligosporus* (Berg *et al*., 2001). The fermentation process has recently been modified and applied on a new barley genotype (Karmose) with a high amyloseand β -glucan content. The modified process has been found to strongly reduce the phytate content while preserving minerals (Eklund-Jonsson *et al*., 2006) and also lower the glycemic index of barley tempeh.

The aim of the study is to prepare the barley tempeh through Koji fermentation by *Rhizopus oligosporous* G-10. The protease and in vitro protein digestibility of unfermented and fermented barley were study.

2. Material and Method

2.1. Culture

The *Rhizopus oligosporous* G-10 was procured from Microbiology Lab, Food and Biotechnology Research Centre, PCSIR Labs Complex Lahore. The strain was grown on PDA agar slant (Oxoid) for 48 h at 30 ^{o}C. The culture was then preserved at 4 ^{o}C and further shifting on the PDA slant at the interval of 30 days to keep them viable. The pH 5 of the medium was adjusted with 1N HCl / NaOH before sterilization at 121 ^{o}C for 15 min.

2.2. Inoculum preparation

Inoculum was prepared by transferring a 10 ml of sterilized distilled water in 48 hour old slant of *Rhizopus oligosporous* G-10. The spores were taken under the sterile

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conditions and inoculum used in this study has a concentration of 10^7 spores / ml.

2.3. Substrates

The substrate (Barely) which is used in this work was obtained from the Local Market (Lahore, Pakistan).

2.4. Solid-state fermentation

The Berg method (2001) for the barely tempeh was used with slightly modification. Hundred grams of dry barely seeds were soaked for 8 hour in the 1L glass beaker which was containing the 500 ml of distilled water. Dehulled the soaked barely by manually and then boiled for 15 minute by adding the 1% glacial acetic acid and 1% sodium chloride to avoid the contamination. The boil water drained off and barley was kept in oven at 80 ^oC for 5 minutes to get the required moisture level for the growth of the strain. Then prepared spore suspension of 1% was used to inoculate the room temperature boiled barley and packed in pre- holed 12 x 12 cm^2 polythene bags for barely cake formation at 30 ^oC for 36 hour . All the experiments were conducted in triplicate.

2.5. Process parameters

The various parameters were studied in koji fermentation of barley by *Rhizopus oligosporous* G-10.The various soaking time of barley in distilled water (4, 6, 8, 10, 12, and 14 hours), boiling time (5, 10, 15, 20, 25 and 30 minute), inoculum size (0.5, 0.75, 1, 1.25, 1.5 and 2 %), incubation temperature (25, 30, 35 and 40 ^0C) and different time interval of koji fermentation (24, 36, 48 and 72 h)**.**

2.6. Crude extracts

Triplicate fermented barely (10 g) were mixed with 100 ml of 0.05 M phosphate buffer (pH 5) and homogenized in a blender machine (Eyela,Japan). The homogenized mixture were kept at room temperature for 30 min with frequent stirring, and then centrifuged at 10000 rpm for 10 min. The supernatant was used as crude enzyme extract.

2.7. Protease activity

Protease activity was determined by the method of Yang and Huang (Yang SS and Huang CI, 1994). The reaction mixture containing 2 mL of 1 % casein solution in 0.05 M phosphate buffer (pH 5) and 1 mL of enzyme solution were incubated at 60 ^oC for 15 min and the reaction was then stopped with the addition of 3 mL of 10 % trichloroacetic acid. After 10 min the entire mixture was centrifuged at 10000 rpm for 10 min at 4 ^oC and absorbance of the liberated tyrosine was measured with respect to the blank at 280 nm. One proteolytic unit (U) was defined as the amount of the enzyme that releases 1 µg of tyrosine per min under assay conditions.

2.8. Protein Digestibility

The *in vitro* protein digestion (IVPD) of barley flour was determined by using the Borchers (Ekpenyong TE and Borchers RL, 1979). A multienzyme technique was used for studied protein digestibility. About 2.60gm of barley flour was dissolved in 50ml of distilled water to give the amount of sample to contain 6.25 mg protein/ml and kept at 5^oC for 1hr. The pH of the sample suspension was adjusted to 8 with 0.1 N HCl / 0.1 N NaOH. Then 5ml of multienzyme mixture (1.6 mg /ml trypsin, 3.1mg /ml chemotropism and 1.3 mg /ml peptidase) were added to the sample suspension with constant stirring at 37 ^oC for 10 min. After incubation the pH of the sample suspension was measured and protein digestibility was calculated by formula IVPD=210.464-18.10X, where X is pH after 10 min digestion.

3. Result and Discussion

In the present study deals with soaking of raw barley in distilled water before Koji fermentation to minimize the hardness effect of barley to improve the growth of *Rhizopus oligosporous* G-10. The results were indicated that the maximum units (78.15 IU/gm) of protease were found after the 8 hour soaking of barley (Fig. 1). The researchers were reported that soaking of the cowpea reduced the antinutritional factors such as protease inhibitors, tannins, phytic acid and flatus-producing oligosaccharides (Ibrahim *et al*., 2002). The soaking increased lysine availability by 21% and 22% for maize and maize-cowpea mixtures in fermentation process was reported by (Nche *et al*., 1995). The soaking in distilled water and $NaHCO_3$ solution significantly reduced the contents of total free phenolics (85–88%) compared to raw seeds (Vijayakumari *et al*., 1995). In this study, soaking has considerable effect on the growth of the strain in barley tempeh.

The cooking has a significant effect on the barley koji fermentation by *Rhizopus oligosporous* G-10. The maximum protease units (80.15 IU /gm) were observed at 15 minutes boiling (Fig. 2). The African yambean was soaked in water for 24 hour and 30 min boiled and reduced the hardness (Marshall *et al*., 2006). The researcher reported that cooking of soaked cowpea further improved lysine availability by 68% and 31% for maize and maize-cowpea mixtures (Nche *et al*., 1995). Cooking has decreased the levels of lectin and trypsin inhibitor in fermented bean (Ana *et al*., 1998). In fermentation of cooked soybeans, proteases, lipases and phytases were produced and due to enzymatic degradation of macromolecules into of lower molecular weight and moist heat have destroying protease inhibitors and expose up the protein structure (Steinkraus *et al*., 1983; Nout and Rombouts , 1990).

The optimum level of inoculum is crucial in fermentation process; low density gives insufficient biomass and high density produces too much biomass and resulting depletion of the nutrients. The results indicated that 1% inoculum gave maximum units of protease (88.10 U/gm) (Fig.3). The spores suspension (1×10^6 spores/ml) was used as inoculum for tempeh from hardened chickpeas with *Rhizopus stolonifer* (Reyes-Moreno *et al*., 2000).

In the present work, the temperature has a significant effect on the growth of *Rhizopus oligosporous* G-10 in koji fermentation of barley for dense mycelia growth. A maximum unit (98.52 U/gm) of protease at 30^oC (Fig.4) is noticed. The optimum temperature of 35.8 ^oC was required for chickpea tempeh formation with *Rhizopus stolonifer* (Reyes-Moreno *et al*., 2000). It was reported that 30^oC was optimum temperature for douchi formation with mould fungus (Chuanlai *et al*., 2005). The inoculation of beans with *Rhizopus oligosporus* at various temperatures

followed by incubation at 30 ^{o}C resulted in both increased and decreased periods for the lag phase of fungal growth (Reu *et al*., 1995).

The time optimization in Koji fermentation is important for maximum mycelia growth and to avoid the stage of the sporulation in tempeh of barley with *Rhizopus oligosporous* G-10. The results of the study indicated that the maximum unit (99.52 U/gm) of protease was found at 36 hour of incubation time (Fig.5). The optimum time of 42.7 hour at 35.8 ^{o}C was reported for the chick pea tempeh with *Rhizopous stolonifer* by the researchers (Reyes-Moreno *et al*., 2000).

The results of the Omafuvbe *et al*, (2004) study indicated that the relative proteolytic activity in the fermenting soybean increased at 48 h of fermentation at 30- 40 ^{0}C. The workers reported that in fermented rice increased in protein level was observed at 24 h of the fermentation (Nnam and Obiakor, 2003). The genus *Rhizopus* secreted enzymes like protease in the tempeh from the substrates like soyabean, wheat, sorghum (milo), oats, rye, barley and corn which improved the quality of the tempeh (Kathleen *et al*., 1993; Waraw Krausong and Yoshikitani, 2005).

The *in vitro* protein digestibility of fermented and unfermented barley was found 62.56% and 28.55% (Table.1). Fermentation was found to create a significant effect on *in vitro* protein digestibility of barley tempeh. The proteolytic enzyme increased the protein digestibility, which was secreted in fermentation. Similar findings show that microorganisms were found to produce proteolytic enzymes during fermentation, which dissociate and degrade proteins, rendering them more accessible to proteases, and hence increased digestibility (Yagoub, 2003; Khaterpaul and Chauhan, 1991; Aderibigbe and Schink ,1990).

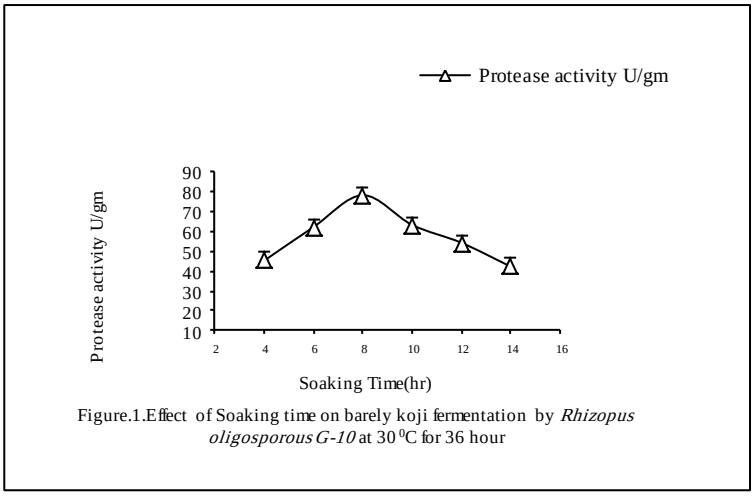

Figure.1.Effect of Soaking time on barely koji fermentation by *Rhizopus oligosporous G-10* at 30 ^{0}C for 36 hour

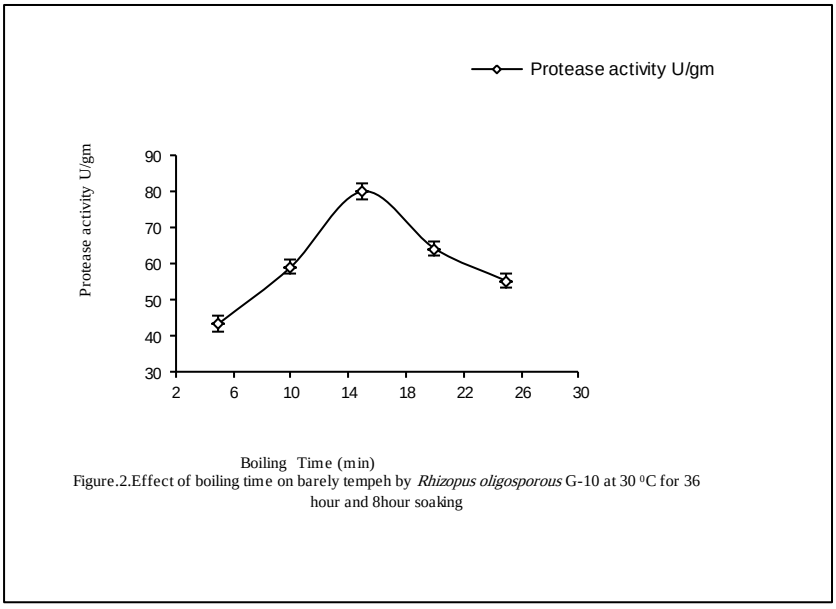

Figure.2.Effect of boiling time on barely tempeh by *Rhizopus oligosporous* G-10 at 30 ^{0}C for 36 hour and 8hour soaking

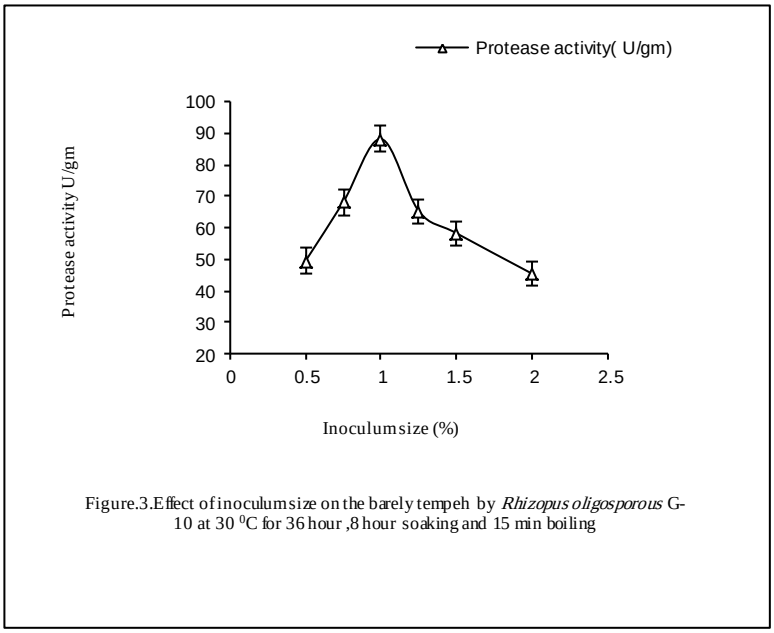

Figure.3.Effect of inoculum size on the barely tempeh by *Rhizopus oligosporous* G-10 at 30 ^{0}C for 36 hour ,8 hour soaking and 15 min boiling

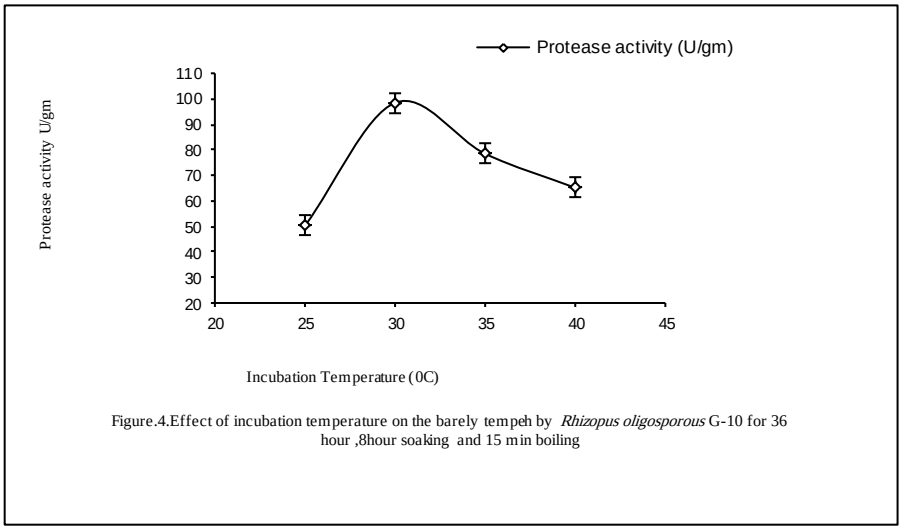

Figure.4.Effect of incubation temperature on the barely tempeh by *Rhizopus oligosporous* G-10 for 36 hour ,8hour soaking and 15 min boiling

Table 1.In vitro protein digestibility of fermented and unfermented barley. Values are the average of three replicates. ±denotes the standard deviation among triplicates.

Time (minute)	pH	*In vitro* Protein Digestibility (%) Unfermented Barley Flour	Fermented Barley Flour
10	7.0	24.45 ± 0.45	48.36 ± 0.55
	7.2	25.32 ± 0.47	49.65 ± 0.54
	7.4	25.67 ± 0.50	50.85 ± 0.57
	7.6	26.35 ± 0.49	52.35 ± 0.58
	7.8	27.85 ± 0.48	57.98 ± 0.58
	8.0	28.55 ± 0.55	62.56 ± 0.75
	8.2	26.24 ± 0.48	54.48 ± 0.59
	8..4	25.26 ± 0.34	47.67 ± 0.47
	8.6	24.71 ± 0.32	45.65 ± 0.46
15	7.0	24.35 ± 0.45	46.36 ± 0.48
	7.2	24.82 ± 0.46	48.67 ± 0.52
	7.4	25.07 ± 0.45	48.54 ± 0.56
	7.6	25.75 ± 0.48	51.55 ± 0.53
	7.8	26.75 ± 0.44	54.95 ± 0.54
	8.0	27.50 ± 0.51	56.64 ± 0.65
	8.2	26.04 ± 0.38	51.48 ± 0.50
	8..4	25.06 ± 0.32	45.57 ± 0.44
	8.6	24.11 ± 0.30	43.65 ± 0.42
20	7.0	23.12 ± 0.35	44.36 ± 0.43
	7.2	24.10 ± 0.40	46.35 ± 0.50
	7.4	24.47 ± 0.45	47.57 ± 0.52
	7.6	24.95 ± 0.41	48.51 ± 0.51
	7.8	25.55 ± 0.44	50.65 ± 0.53
	8.0	26.70 ± 0.50	54.44 ± 0.56
	8.2	26.04 ± 0.38	51.48 ± 0.50
	8..4	24.56 ± 0.31	45.07 ± 0.42
	8.6	24.01 ± 0.30	43.15 ± 0.40

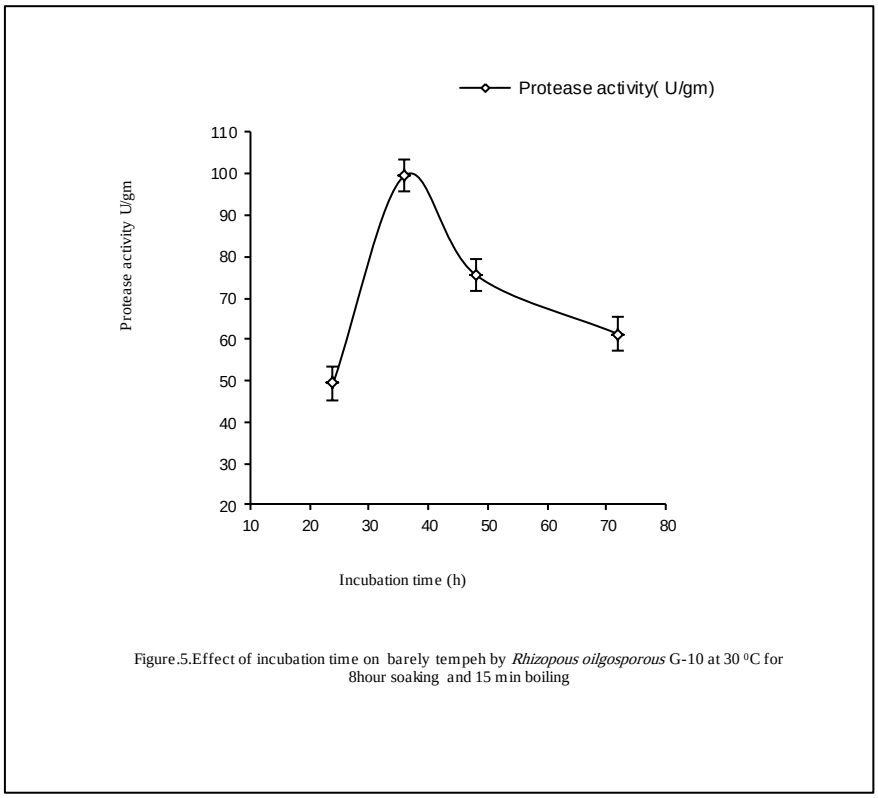

Figure.5.Effect of incubation time on barely tempeh by *Rhizopous oilgosporous* G-10 at 30 ^{0}C for 8hour soaking and 15 min boiling

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A Regulatory Approach Caused by Cold Acclimation and Arsenic on the Impairment of Root Growth of Rice (*Oryza sativa*)

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Abstract

Development of root growth is impaired by environmental and chemical effectors although the mechanism is not known. Here, we used pot experiment for cultivation of rice (*Oryza sativa*) to identify the factors involved in impaired growth of root and to explore the effects of cold acclimation and arsenic on the amylase and urease activity in root. The amylase activity was increased whenever exposed to cold for 48h and 72h while mild effect occurred after 24h duration. Acclimation to cold in the presence of different concentrations of Na_2HAsO_4 also increased the amylase activity effectively than cold exposure alone; however, higher concentration of Na_2HAsO_4 is potentially involved in this respect. Conversely, root urease activity was impaired and reduced by cold for the above mentioned times. The reduced urease activity was also caused by the combined effect of cold and arsenic compound. Increased amylase activities in response to cold and arsenic might be involved in the survival process. The two effectors are also involved in the impairment of reduced uptake of urea because of the reduced urease activities. The results clearly demonstrate that both effectors produced an adverse environment where the growth and development of roots are impaired.

Keywords: Cold acclimation, arsenic, root growth, adaptive response.

1. Introduction

Rice (*Oryza sativa*) is one of the most important cereal crops in the world. It is a principal food in developing and developed countries. Only 5% of the total production of rice is used in processed foods, industrial products and alcoholic beverages, while 95.0% of world rice production is consumed as an unprocessed food (Rahman *et al*., 2007). Therefore, the development of this plant is an important aspect in plant metabolism as well as in the metabolism of the other heterotrophic organisms. Root development is impaired either by environmental or chemical factors. Temperature fluctuation is a common phenomenon of the atmosphere and is involved in changes of various metabolic functions (Janska *et al*., 2010). It has been revealed that roots and other protected parts are less cold hardy than the aerial parts of the plant (Havis, 1976; Pellet, 1971). Therefore, it is presumably assumed that cold acclimation may have the role in the development of plant roots (Räisänen *et al*., 2009). Evaluation of the cold hardiness of roots during winter is difficult due to the frozen soil and the lack of reliable methods for assessing freezing damage (Chen *et al*., 1983).

Development of plant growth is one of the biological processes mediated by the coordination of the metabolic processes catalyzed by different enzymes (Renaut *et al*., 2006). For example, urease is involved in the degradation of urea to CO_2 and NH_4^+, the higher the degradation of this urea (N-fertilizer), the higher the formation of NH_4^+ thereby utilization of nitrogen in plant (Haque *et al*., 2010; Mérigout *et al*., 2008). Similarly, amylase is involved in the degradation of amylose to monosaccharide. Amylose is an essentially linear molecule composed of α (1- 4)-linked glycosidic chains (Nishi *et al*., 2001). Therefore, the utilization of these products and their involvement is an important aspect in plant metabolism. Efficiency of nitrogen use is evaluated in terms of development of efficient photosynthetic machinery involving biosynthesis of proteins which mediates the various metabolic steps in the chloroplast (Younis *et al*., 2008). The other aspects of the nitrogen use efficiency are the overall leaf growth, canopy development, light interception and contribution to total photosynthesis (Yildirim *et al*., 2007). All these metabolic activities eventually determine the biomass of plant. The biological processes regarding the development of the plant are directly or indirectly modulated by natural heavy or light elements. Arsenic (As) has been identified to be toxic to the living organisms (Li *et al*., 2007). Prolonged exposure of arsenic has detrimental effects in tissues. It may impair the glycolysis as well as the oxidative processes (Tchounwou *et al*., 2003) and causes different types of pathogenic syndromes in rodents and other organisms. More than 40 million people worldwide are at risk from drinking As-contaminated groundwater (Nordstrom, 2002), and chronic inorganic As poisoning has reached a massive scale in Bangladesh and West

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Bengal, India (Bhattacharjee, 2007). In these countries, As contaminated ground water is also widely used for irrigating crops during dry season for rice production in Bangladesh resulting in arsenic accumulation in soils and elevated arsenic uptake by crops (Meharg and Rahman, 2003; Alam and Sattar, 2000). Elevated arsenic accumulation in rice has the potential to become a new disaster for the population in Southeast Asia (Meharg, 2004). Arsenic concentration in rice grain is often high enough to cause concern even in uncontaminated soils containing background levels of As, because paddy rice appears to be particularly efficient in As assimilation compared with other cereal crops (Williams *et al*., 2007). It is therefore crucial that the mechanism of arsenic accumulation in rice is understood to counteract this widespread contamination of the food chain. The roots of plants are involved in the acquisition of water and nutrients, anchorage of the plant, synthesis of plant hormones and storage functions. Therefore, to fully understand the morphogenesis of roots, it is necessary to analyze the activity of amylase and urease to clarify their role in paddy development and how these enzymes are regulated by environmental adverse effectors like cold and toxic arsenic.

2. Materials and Methods

2.1. Soil collection and pot preparation

The soil was collected from the rice field of Rajshahi University Campus, Bangladesh and kept in several plastic pots. The unwanted materials like stones, gravels, pebbles, plant roots, etc. were removed from the bulk soil. For this experiment, four plastic pots were used; the size of each pot was 70 cm in diameter and 24 cm in height. An adequate amount of soil was taken in each plastic pot. Then sufficient amount of water was poured into each pot and kept for overnight and mixed well. Then the pots were ready for seedling of germinated rice.

2.2. Seed germination

For the germination of seeds (*Oryza sativa*), the following points were carried out: (i) the strongest seeds were selected; the seeds were added to water and floating seeds were discarded; (ii) the seeds were kept in water with temperature below 37^0C overnight; (iii) the seeds were swollen by water absorption and were expected to be effective for germination; (iv) the seeds were seeded in the pots prepared with soil and the efficiency of seed germination was about 90%.

2.3. Cold acclimation and arsenic treatment

After 10 days of germination, the four different pots were described as control, cold, arsenic (1 mM) plus cold and arsenic (10 mM) plus cold. Control pot was used for 24h, 48h and 72h treatments in the room temperature without cold acclimation. The second pot was used for 24h, 48h and 72h duration in the cold chamber and given cold exposure (4~8^0C) with full aeration. In the third pot, paddies were treated with arsenic (Na_2HAsO_4. $7H_2O$, BDH Chemical Ltd.) (1 mM) and kept similarly in cold for 24h, 48h and 72h in the cold chamber. The fourth pot containing paddy was similarly treated with arsenic (10 mM) and kept in cold for 24h, 48h and 72h in the cold chamber. After 10 days of germination, paddies were ruptured consecutively from each pot and the different parts of paddy including root were sampled carefully.

2.4. Assay of amylase and urease activity

One g of paddy root was placed into a mortar with pestle which was kept on ice. It was homogenized with 12 ml of distilled water and centrifuged with 8000 rpm for 10 minutes. The supernatant was collected and used as crude extract. The amylase and urease activities in the crude extract were assayed by the method described by Jayaraman (1981). For amylase and urease activity, 1.0 and 0.1 ml of the crude extract were used respectively. The enzyme activities in root extracts were expressed as µmole/min/mg of protein.

2.5. Assay of protein content in root extract

Roots (1 g) were homogenized with pre-cooled water and were centrifuged at 8000 rpm for 10 min. The supernatants from each root homogenate were used as crude extract for assay of protein by using 100 µl extract. The protein content in root was determined by the procedure of Lowry *et al*. (1951). Briefly, alkaline solution was prepared by mixing 50 ml of alkaline Na_2CO_3 solution (2% Na_2CO_3 in 0.1N NaOH) and 1.0 ml of copper-sodium potassium tartarate solution (1 g sodium potassium tartarate and 0.5 g $CuSO_4$. $5H_2O$ were dissolved in 100 ml distilled water). Hundred µl of tissue extract was taken to the test tube and made up to 1 ml with distilled water. For blank, 1 ml water was used in place of tissue extract. Five ml alkaline solution was added to each tube and mixed well. The tubes were allowed to stand for 10 min at room temperature and 0.5 ml of diluted FCR (Commercial FCR was diluted with equal volume of water) was added and mixed well. After 30 min, the absorbance was taken at 650 nm against the blank. The protein content in each root extract was calculated from the standard graph of bovine albumin (1 mg/ml) and is expressed as g/100 g of root weight.

2.6. Statistical analysis

Results of the experiments were expressed as mean and standard error of different groups. The differences between the mean values were evaluated by ANOVA followed by paired *t*-test using SPSS software.

3. Results and Discussion

3.1. Effect of cold and Na_2HAsO_4 on amylase activity

To examine the role of cold exposure and arsenic treatment on the regulation of amylase activity in root of paddy, the plants in the pot were exposed to cold for 24h in the cold chamber. As shown in figure 1**,** the amylase activity for control was 0.0787 ± 0.0099 µmole while for cold treatment; the value was 0.0706 ± 0.0021 µmole$min^{-1}mg^{-1}$ of protein. The activity in root extract was reduced by 10.29% in response to cold only. Whenever, the paddy was exposed to different concentrations of arsenic and cold, the different amylase activities were observed. In this case, the paddy exposed to 1 mM Na_2HAsO_4 and cold had the enzyme activity 0.1538 ± 0.0123 µmole$min^{-1}mg^{-1}$ of protein after 24 hours of treatment. The results indicated that the activity in paddy root in response to

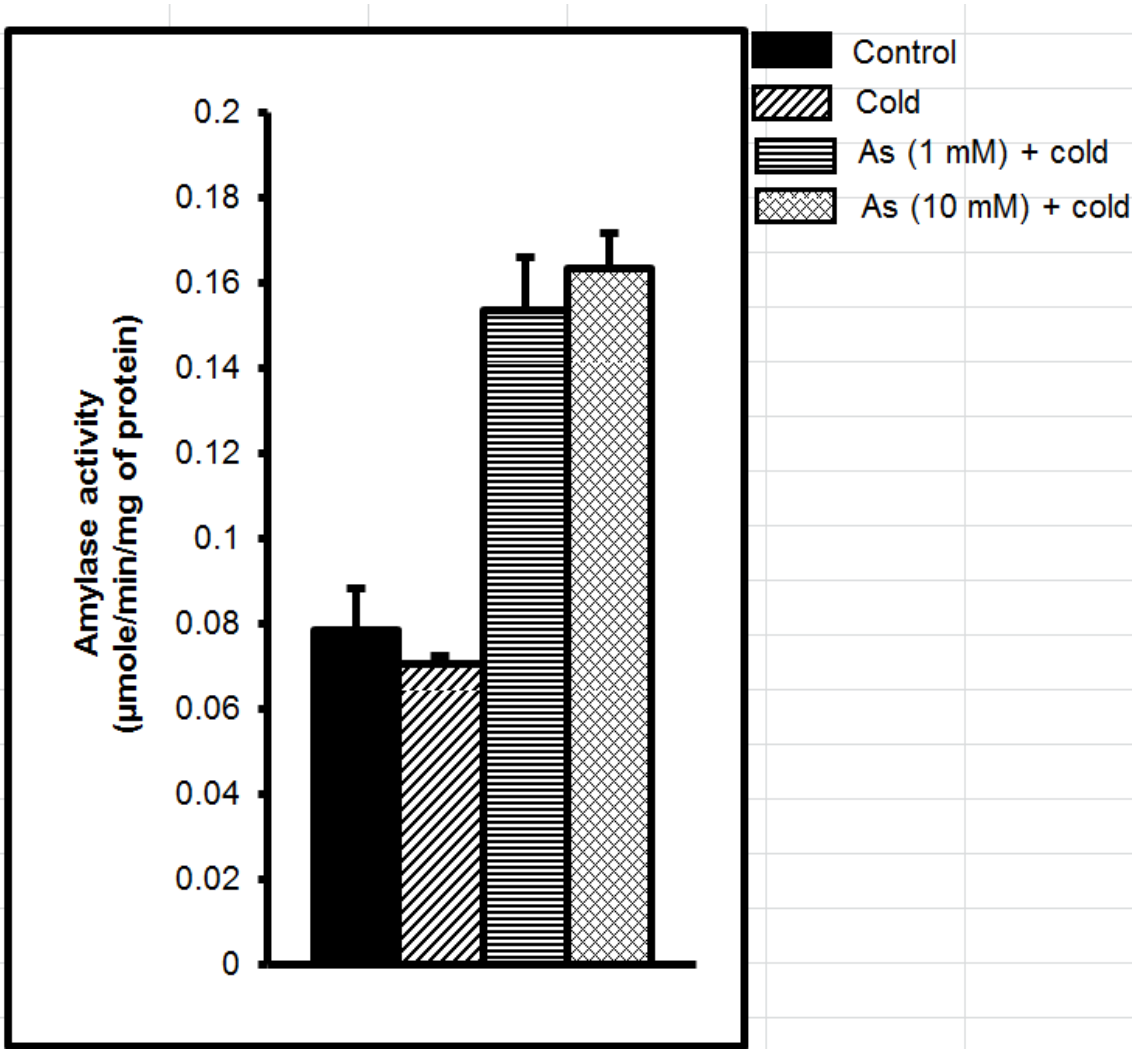

Figure 1. Effect of cold and Na_2HAsO_4 on amylase activity in roots after 24h of treatment. The paddy was treated with different concentrations of arsenic (1 and 10 mM) and kept for 24h in the cold. The paddies in another pot were exposed to cold for 24h only in the cold chamber. Control paddy was similarly used without cold exposure and arsenic treatment. The data are means ± SE for 3 individual measurements in each group.

arsenic had been found to be influenced by 95.42% when compared to control. In addition, the enzyme activity was enhanced by 117.84% when compared to cold exposed paddy. Therefore, it is presumably assumed that short term cold exposure has little impairment on root growth and development; conversely, cold acclimation in presence of arsenic induces a severe effect and stimulates amylase activity in root. In response to 10 mM Na_2HAsO_4 and cold, the amylase activity was found to be 0.1635 ± 0.0085 µmolemin^{-1}mg^{-1} of protein. The results indicated that the activity was increased by 107.75% when compared to control and 131.58% compared to cold exposure only, however, higher concentration of arsenic produces the increased efficiency on enzyme activity. It is evident, both cold acclimation and arsenic are severely involved in inducing the adverse environment where the paddy survives for their growth.

To find the optimum effect of cold acclimation on amylase activity in paddy roots, the extended time was 48h (Fig.2). The cold exposed paddy had amylase activity of 0.0890 ± 0.0116 µmole while for control paddy; the activity was 0.0415 ± 0.0029 µmolemin^{-1}mg^{-1} of protein. The results showed that the activity in root had been increased by 114.45% when they were exposed to cold for 48h when compared to control. The paddy exposed to cold and arsenic (1 mM Na_2HAsO_4) for 48h had enzyme activity of 0.0485 ± 0.0028 µmolemin^{-1}mg^{-1} which indicated that arsenic treated paddy had showed increased activity (16.86%) when compared to control. Moreover, the enhanced enzyme activity (45.50%) was observed in arsenic and cold exposed paddy when compared to cold exposed paddy only. Paddy exposed to cold and arsenic (10 mM Na_2HAsO_4) had urease activity of 0.0380 ± 0.0083 µmolemin^{-1}mg^{-1} of protein. The activity in response to higher dose of arsenic and cold was reduced by 8.43% and 57.30% when compared to control and cold exposed paddy, respectively. The results in figure 2 seem to indicate that the enzyme activities were affected by both cold acclimation and arsenic; however, cold acclimation was critically involved to increase the activity.

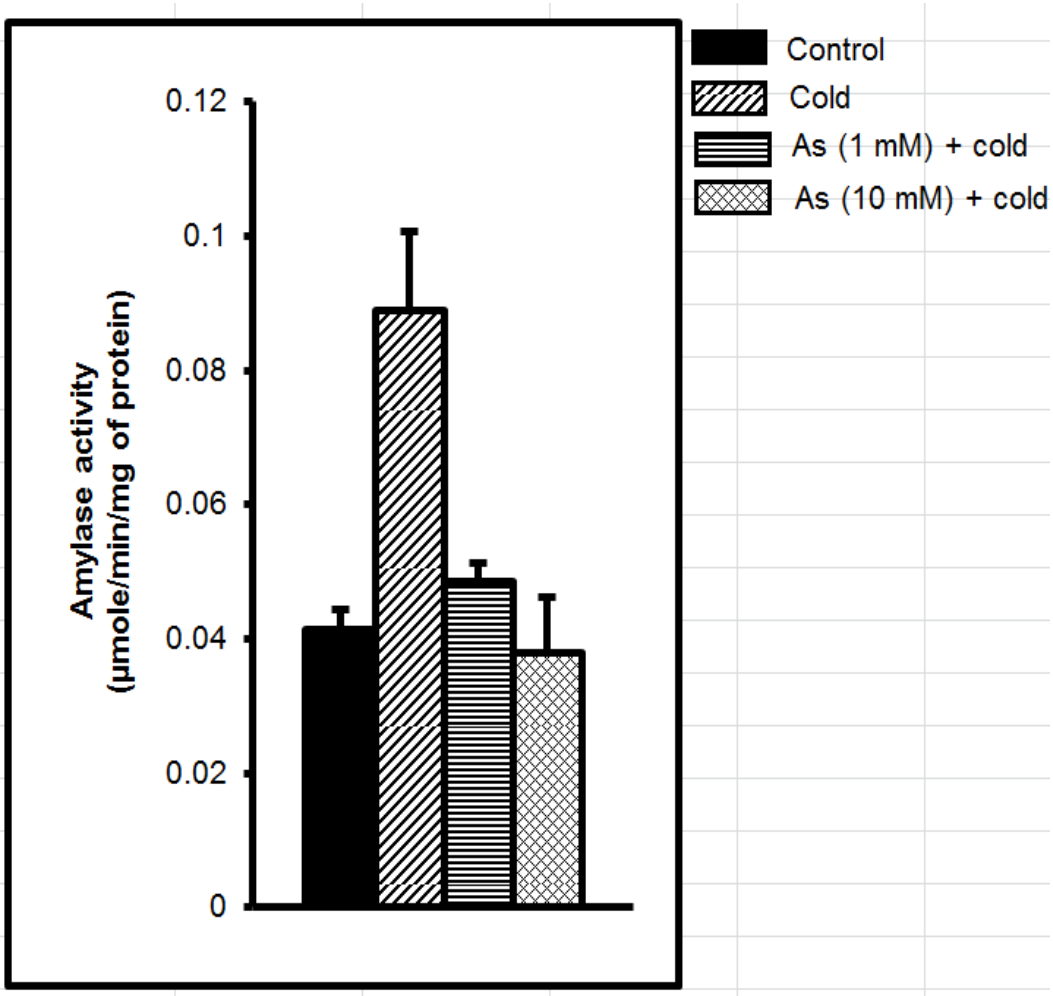

Figure 2. Effect of cold and Na_2HAsO_4 on amylase activity in roots after 48h of treatment. The paddy was treated with different concentrations of arsenic (1 and 10 mM) and kept for 48h in the cold. The paddies in another pot were exposed to cold for 48h only in the cold chamber. Control paddy was similarly used without cold exposure and arsenic treatment. The data are means ± SE for 3 individual measurements in each group.

After 72 hours of treatment, the amylase activities of different types of treated paddy were estimated as 0.0621 ± 0.0046 µmole for control and 0.0698 ± 0.0065 µmole for cold treatment (Fig.3). On the other hand, whenever they were exposed to cold and arsenic (1 mM), the root activity was 0.0757 ± 0.0005 µmolemin^{-1}mg^{-1} .Similar increased effects on root enzyme activities (12.39%) were obtained whenever the plants were exposed to cold while in presence of arsenic, cold acclimation causes similar increased effects on activity by 21.90% when compared to control. However, compared to cold acclimated paddy, arsenic in presence of cold was found to be involved to increase the root enzyme activity by 8.45%. The amylase activity in response to 10 mM Na_2HAsO_4 and cold was found to be 0.0630 ± 0.0059 µmolemin^{-1}mg^{-1} of protein which shows that the enzyme activity was similarly increased by 1.44% when compared to control while reduced by 9.74% as compared to cold exposed paddy. The results indicate that prolonged cold acclimation might be involved in reducing the amylase activity in response to arsenic since growth and development of paddy are retarded after 72h of treatment.

3.2. Effects of cold and Na_2HAsO_4 on urease activity

As shown in figure 4, the urease activities in roots of treated paddy were recorded to determine the effect of cold and arsenic on root growth. After 24 hours of treatment, the root urease activities were estimated as 0.5430 ± 0.0137 µmole for the control and for cold treated paddy, the value was 0.1940 ± 0.0076 µmolemin^{-1}mg^{-1} of protein. Cold acclimation caused a significant decrease in urease activity by 64.27% when compared to the control. However, when paddies were exposed to cold and arsenic (1 mM Na_2HAsO_4) for 24h, the different urease activity was observed and found to be 0.3080 ± 0.0258 µmolemin^{-1}mg^{-1} of protein. The results demonstrate that the urease activity of paddy had been similarly decreased by 43.27% when compared to the control, however, in comparison to cold acclimated paddy, the increased effect (58.76%) on root activity was observed in response to arsenic and cold. The paddy exposed to 10 mM arsenic and cold had urease activity of 0.1990 ± 0.0107 µmolemin^{-1}mg^{-1} showing that the enzyme activity was reduced by 63.35% and increased by 2.57% when compared to control and cold exposed paddy, respectively.

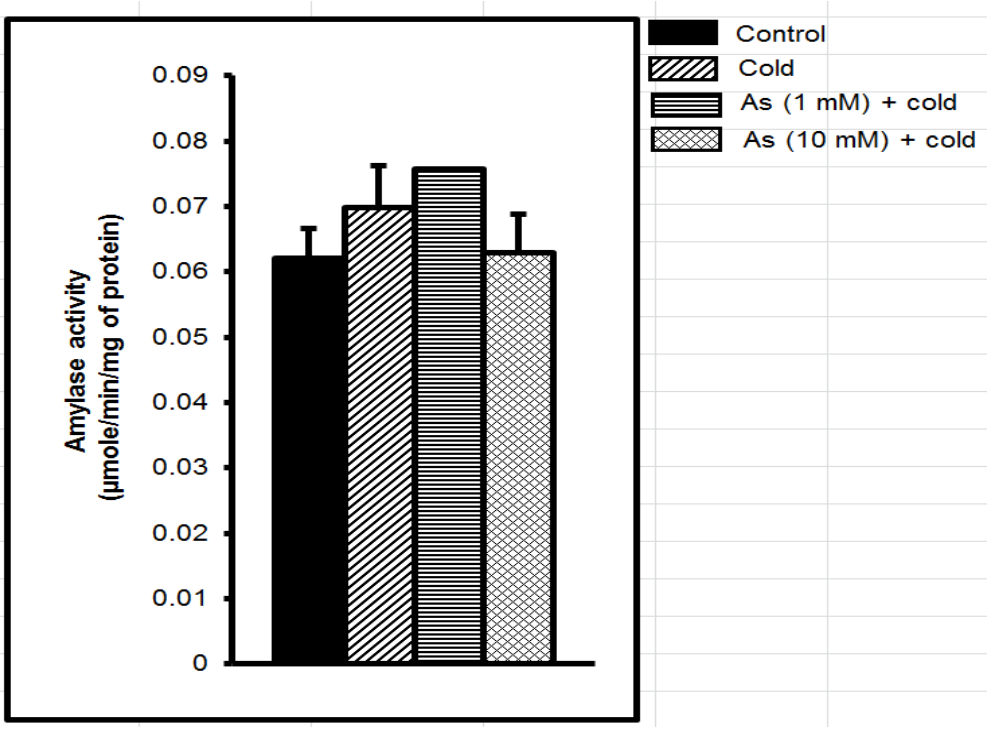

Figure 3. Effect of cold and Na_2HAsO_4 on amylase activity in roots after 72h of treatment. The paddy was treated with different concentrations of arsenic (1 and 10 mM) and kept for 72h in the cold. The paddies in another pot were exposed to cold for 72h only in the cold chamber. Control paddy was similarly used without cold exposure and arsenic treatment. The data are means ± SE for 3 individual measurements in each group.

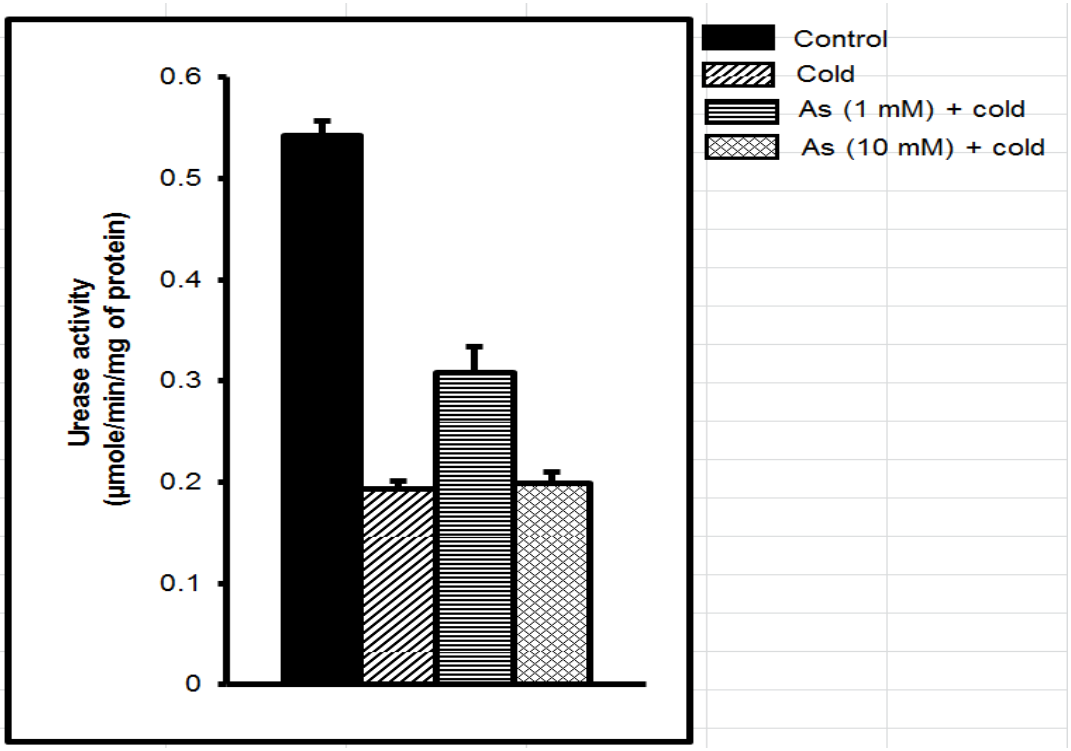

Figure 4. Effect of cold and Na_2HAsO_4 on urease activity in roots after 24h of treatment. The paddy was treated with different concentrations of arsenic (1 and 10 mM) and kept for 24h in the cold. The paddies in another pot were exposed to cold for 24h only in the cold chamber. Control paddy was similarly used without cold exposure and arsenic treatment. The data are means ± SE for 3 individual measurements in each group.

The urease activity might be regulated by the variation of temperature and be strictly followed by the availability of urea in the soil.

To examine the effect of cold on urea induced urease activity, the plants were exposed to cold for 48h along with the combined effect of cold and arsenic. As shown in figure 5, the root urease activities of treated paddy were found to be 0.4950 ± 0.0360 µmole for the control and 0.1180 ± 0.0037 µmole for cold treatment. It was found that the urease activity of paddy root had been reduced by 76.16% for cold acclimation. The paddies exposed to cold and arsenic (1 mM Na_2HAsO_4) had urease activity of 0.1330 ± 0.0077 $\mu molemin^{-1}mg^{-1}$ of protein showing the reduced effect on enzyme activity (73.13%) when compared to control. However, compared to cold exposed paddy, the urease activity was increased by 12.71%. In response to arsenic 10 mM and cold, root urease activity was 0.0670 ± 0.0040 $\mu molemin^{-1}mg^{-1}$ of protein. Addition of higher dose of arsenic also causes the lower effect (86.46%) when compared to control; however, it was lower than the cold exposure alone. Therefore, cold acclimation was directly involved to reduce the urease activity and the effect might be changed in response to the availability of arsenic in the soil. The reduced effect (43.22%) on root activity was occurred by 10 mM arsenic and cold compared to cold acclimated paddy. Therefore, the impairment on urease activity caused by cold acclimation is associated with the administration of arsenic.

Figure 6 shows the effect of cold and different concentrations of arsenic on urease activity of paddy after 72 hours of treatment. Paddies treated with cold had root urease activity 0.2130 ± 0.0348 µmole, whereas 0.1350 ± 0.0127 $\mu molemin^{-1}mg^{-1}$ of protein were recorded for 1 mM Na_2HAsO_4 and cold treatment. The root urease activity of paddy for the control treatment was 0.7240 ± 0.0232 $\mu molemin^{-1}mg^{-1}$ of protein. These results indicate that the root urease activity of paddy had been reduced by 70.58% for cold treatment and 81.35% for arsenic and cold treatment as compared to control. However, compared to cold exposed paddy, the activity was also reduced by 36.61%. Cold acclimation similarly causes the reduced activity in prolonged time and they survive in such critical environment either by lower uptake of soil urea or by other phenomenon. Addition of higher dose of arsenic (10 mM), also seems to be involved in reducing the enzyme activity. Here, the enzyme activity was 0.0400 ± 0.0013 $\mu molemin^{-1}mg^{-1}$ of protein. The results also show that the urease activity was reduced by 81.22% when the paddies were exposed to cold and arsenic compared to cold acclimation. Moreover, 94.47% reduced activity occurred in response to arsenic and cold as compared to the control. Therefore, it is reasonable that both cold and arsenic creates an adverse environment and may reduce the activity because of the lower uptake of urea in the soil.

In our study, we found that the amylase and urease activity in root of paddy had been affected by arsenic and cold treatment. Although 24h treatment with cold did not increase the amylase activity, however, prolonged exposure affected and enhanced its activity and the reduced activity was considered to be the mild effect of cold. In different studies, it was assumed that cold acclimation induces glucose synthesis in plants (Wanner and Junttila, 1999); therefore, enhanced activity of amylase is correlated to their results. It has been revealed that sugars appear essentially in plant during cold acclimation as shown for example by the inability of an Arabidopsis sucrose synthase mutant to cold acclimation (Welling and Palva, 2006) or the requirement for light in low- non freezing temperature-induced cold acclimation connected to sugar accumulation (Wanner and Junttila, 1999, Yadegari *et al*., 2007). Precise function of sugars is not known, but their high abundance in cold acclimated plants suggest a role in osmoregulation and less abundant sugars might also have a role in cryoprotection or as signaling molecules (Welling and Palva, 2006). There is increasing evidence that chilling causes elevated levels of active oxygen species (AOS), which contribute significantly to chilling damage (Wise and Naylor, 1987). AOS such as superoxide (O^{-}_2), hydrogen peroxide (H_2O_2), hydroxyl radicals (OH^{-}) and singlet oxygen ($^1O_2$), are present in plants in various at 25 ^oC as a result of normal aerobic metabolism. Therefore, accumulation of sugars during cold acclimation might be correlated to the above mechanisms. Heavy metal accumulation in soil and its importance on the morphological, biochemical and cytological aspects of plants have been considered to be the major issue for the development of plant growth by many workers (Abbasi *et al*., 1992; Prakash *et al*., 2004). The results appeared to be affected more severely whenever the pants were exposed to higher arsenic concentration (10 mM). A number of studies demonstrated the reduced growth of plants (including paddy) grown in soil containing high arsenic or when irrigated with water containing high concentration of arsenic (Smith *et al*., 2001). The data presented in the study show that Na_2HAsO_4 in different doses stimulated the enzyme activity possibly by impairing root growth and development. Stoeva *et al*. (2003) also reported that arsenic accumulated mainly in the root system and to a lesser extent in the overgrown organs, inhibits the growth and fresh and dry biomass accumulation. There is, however, contrasting reports showing that the effect of Na_2HAsO_4 on shoot and root growth is similar (Simon *et al*., 1978). It seems likely that the effect of Na_2HAsO_4 on stem and root growth varies depending on the plant species, level of contamination and plant tissue ability to tolerate Na_2HAsO_4. It is therefore, both cold acclimation and arsenic can be regarded as to be the adverse effectors for the plant growth.

The reduced urease activity in root extract in response to cold was reported in our study. The adverse environment created by cold acclimation for prolonged period was made, therefore, nutritional deficiencies and other cellular defects might be happened in paddy. Promotion of growth is impaired during cold acclimation. Therefore, protein synthesis because of the utilization of nitrogenous substances is impaired and as a result, urease activity should be declined. In addition, deficiency of urea in soil is also associated to this lower activity of urease. In soils, urea is rapidly degraded to ammonium and CO_2 by urease, a nickel-dependent enzyme, which amongst others is synthesized and secreted by microorganisms (Watson *et al*., 1994).

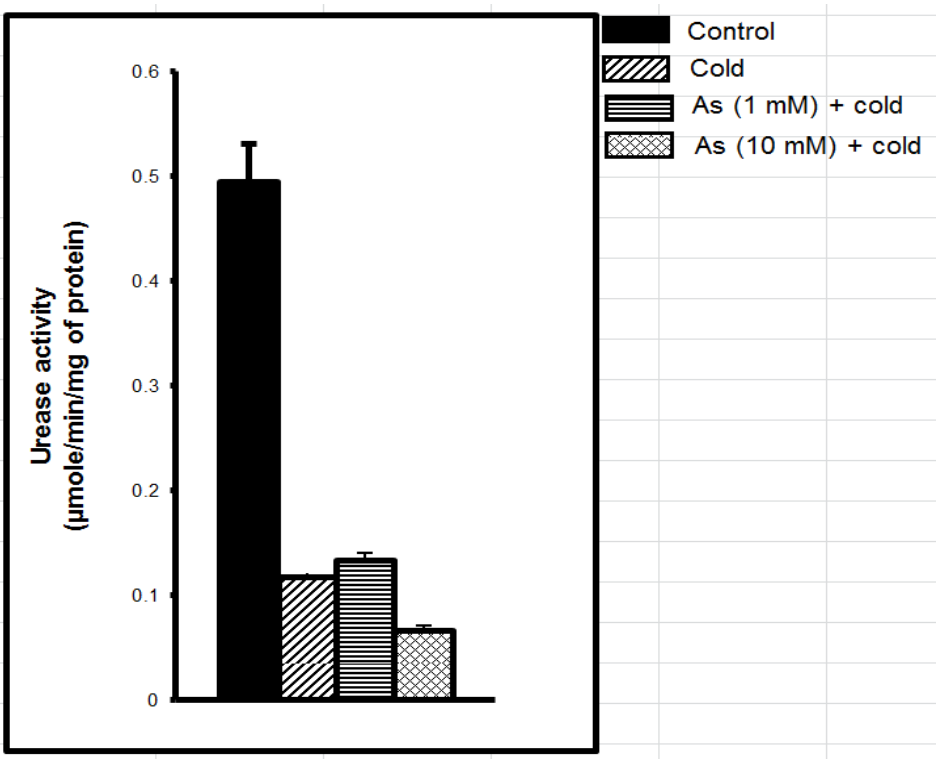

Figure 5. Effect of cold and Na_2HAsO_4 on urease activity in roots after 48h of treatment. The paddy was treated with different concentrations of arsenic (1 and 10 mM) and kept for 48h in the cold. The paddies in another pot were exposed to cold for 48h only in the cold chamber. Control paddy was similarly used without cold exposure and arsenic treatment. The data are means ± SE for 3 individual measurements in each group.

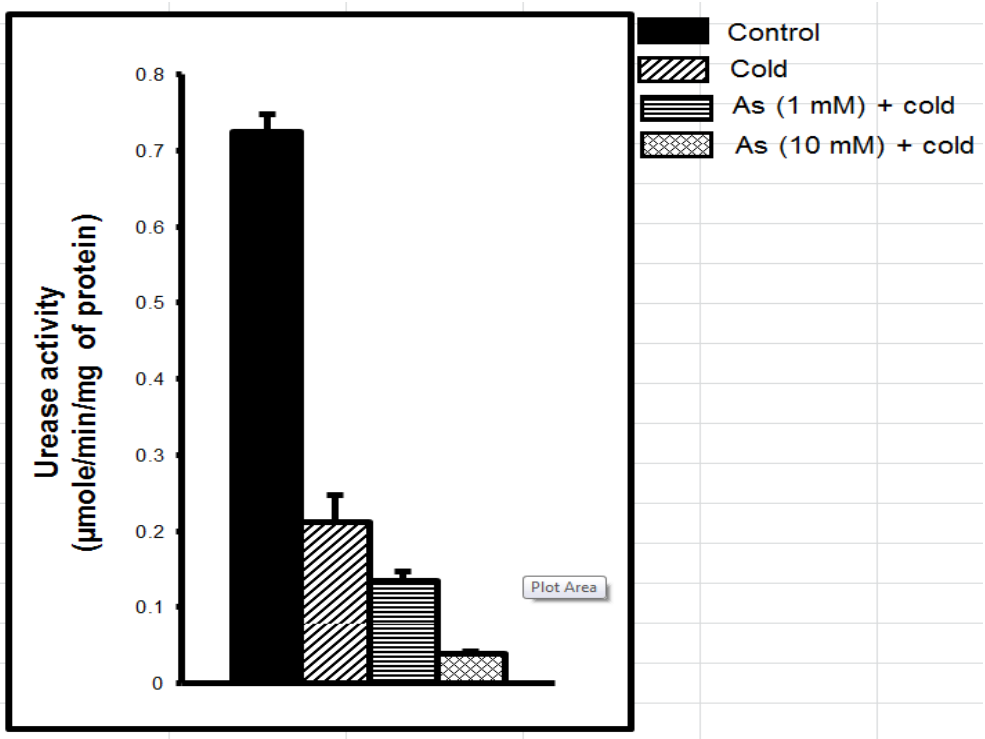

Figure 6. Effect of cold and Na_2HAsO_4 on urease activity in roots after 72h of treatment. The paddy was treated with different concentrations of arsenic (1 and 10 mM) and kept for 72h in the cold. The paddies in another pot were exposed to cold for 72h only in the cold chamber. Control paddy was similarly used without cold exposure and arsenic treatment. The data are means ± SE for 3 individual measurements in each group.

Therefore, the concentration of urea in lakes or natural soils is usually low and ranges between 0.1-3.0 µM (Cho *et al*., 1996), but upto 70 µM in fertilized crop-planted soils. With regard to this very low concentration it was believed that plants take up urea-derived nitrogen mainly in the form of ammonium (Polacco and Holland, 1993). We found that cold acclimation along with Na_2HAsO_4 causes an adverse environment where plants survive for their growth; therefore, the impaired root growth associated with reduced urease activity is possible. Previous study revealed that in woody species, cold hardening of roots is determined by genotype, soil temperature, and moisture (Wildung *et al*., 1973). However, little information on root cold hardiness and development following freezing is available for winter cereals. It has been suggested that roots and the lower portions of the crown of cereals are more susceptible to freezing injury than the leaves and upper crown tissue (Olien and Marchetti, 1976). There was a reduction in shoot and root growth in wheat plants frozen from -10 to -20^{o}C when transplanted to soil (Chen *et al*., 1983). The reduction in shoot growth was probably due to the effect of the lower temperatures on root regeneration. The growth of roots can be influenced by temperature gradients (Fortin and Poff, 1990), mechanical impedance (Barley and Greacen, 1967), aeration (Cannell, 1977) and the roots of adjacent plants (Mahall and Callaway, 1991). A recent study (Li *et al*., 2007) revealed that at higher concentration, arsenic is toxic to most plants. It interferes with metabolic processes and inhibits plant growth and development through arsenic induced phytotoxicity (Marin *et al*., 1993). When plants are exposed to excess arsenic either in soil or in solution culture, they exhibit toxicity symptoms such as: inhibition of seed germination (Abedin and Meharg, 2002); decrease in plant height (Marin *et al*., 1992), reduction in root growth (Abedin and Meharg, 2002), and lower fruit and grain yield (Abedin *et al*., 2002; Kang *et al*., 1996). Both cold acclimation and arsenic cause similar adverse environment, therefore, uptake of nitrogen to the plants is prohibited, utilization of soil urea is also affected because of the lower activity of urease. In these circumstances, a strategy might be developed either by administration of urea or other N-fertilizer. It is, therefore, the regulation of urease activity could be an index for the enhancement of the development of paddy growth.

4. Conclusion

Cold acclimation and arsenic have been found to be involved in the impairment of root development and plant growth. Although several factors might be involved in this respect, however, these two environmental and chemical effectors are found to be predominantly involved. The regulation of these two enzymes in response to cold and arsenic is an important aspect in plant metabolism and might be an index for the species of paddy for their growth during these critical environmental circumstances. Our investigations gave a concept to find a strategy for the survival of this plant species in these critical situations. It is assumed from the results that the impairment of root growth is mediated by the combined effects of these stressful effectors and coordinately to each other.

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Appendix A

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Appendix B

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مجلة علمية عالمية محكمة

المجلة الأردنية للعلوم الحياتية : مجلة علمية عالمية محكمة ومفهرسة ومصنفة، تصدر عن الجامعة الهاشمية و بدعم من صندوق دعم البحث العلمي- وزارة التعليم العالي و البحث العلمي .

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